TAMPEREEN TEKNILLINEN YLIOPISTO
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RÉKA HAJDU-RAHKAMA

BIOOXIDATION OF IRON IN ELEVATED PRESSURES AND PRODUCTION OF IRON OXIDIZING BIOMASS FOR A PILOT-SCALE BIOREACTOR

Master of Science Thesis

Examiners: Professor Jaakko Puhakka and Assistant Professor Aino-Maija Lakaniemi
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ABSTRACT

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Securing the future´s metal demand through traditional metal recovery methods is often economically not viable because of the low metal content of the readily available ores. Although biological metal recovery from low-grade ores can be potential alternative, the recently used approaches such as heap and tank bioleaching still require the extraction and crushing of the ores. Therefore, an environmentally friendly approach that would work with low-grade ores at the natural occurrence of metals known as deep *in situ* bioleaching is under investigation. Studying the pressure tolerance of a mixed acidophilic iron oxidizing microbial community (*Leptospirillum ferriphilum* and *Sulfobacillus* sp.) that could be used in deep *in situ* application was the main objective of this thesis. Furthermore, production of activated carbon-bound iron oxidizing biomass for pilot-sale demonstration of *in situ* bioleaching was also conducted.

Experiments with a pressure reactor (1 L) showed pressure tolerance of the acidophilic culture at 40 bar (with initial 0.3 bar oxygen partial pressure (p_{O2}), while the pressure was induced with N_2 gas) above atmospheric pressure. The 10 bar/min pressure increase/decrease rate was not inhibitory to the iron oxidation activity of the microorganisms. When the elevated pressure was induced with technical air, the highest tolerated pressure where biotic iron oxidation still occurred was +3 bar (p_{O2}=0.63 bar). From the elevated pressures tested, the highest biotic iron oxidation rate (0.78 g/L/d) was obtained at +3 bar, which was approximately half of the rate obtained at atmospheric pressure (1.7 g/L/d) in shake flask cultures. The abiotic iron oxidation rate linearly increased with the increase of oxygen partial pressure. During the biomass production for the pilot reactor, it was shown that the iron oxidation rate decreased as the reactor volume got larger. In order to reach iron oxidation efficiency of 90% took approximately 0.3, 3 and 4 days in the fluidized bed reactor (900 mL), shake flasks (100 mL) and semi-pilot reactor (~600 L), respectively.

This work demonstrated that *in situ* iron oxidation by acidophilic microbial community of this study in culture suspension is possible up to +3 bar (p_{O2}= 0.63 bar). Abiotic iron oxidation in deep subsurface is an option if oxygen can be provided there. To achieve the highest possible iron oxidation rate and maintain the microbial community structure, fully controlled environment (pH, temperature, pressure, mixing, aeration) and continuous operation are required.

PREFACE

This work´s pressure experiments and laboratory-scale biomass productions were carried out in the Laboratory of Chemistry and Bioengineering of Tampere University of Technology, the semi-pilot scale biomass production at the Geological Survey of Finland (GTK) and the biomass maintenance at the BIOMOre chamber located in the Rudna mine of KGHM Polska Miedz. I wish to thank the European Union´s Horizon 2020 research and innovation programme (grant agreement No 642456) for the funding of this study.

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Réka Hajdu-Rahkama

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LIST OF SYMBOLS AND ABBREVIATIONS

AC	activated carbon
Ag^{+}	silver ion
atm	atmospheric pressure
c_{aq}	concentration in aqueous phase
Cl^{-}	chloride ion
Cd^{2+}	cadmium ion
CO_2	carbon dioxide
conc.	concentration
d	day
DO	dissolved oxygen
E_a	energy of activation
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
Fe(tot)	total iron
FBR	fluidized bed reactor
FIGB	ferric iron-generating bioreactor
g	gram
Gt	goethide
Hg^{2+}	mercury ion
mg	milligram
halite	sodium chloride
HRT	hydraulic retention time
ISL	*in situ* leaching
J	Joule
K	potasssium
k	constant
kJ	kilo Joule
L	liter
M	mesophilic
Mg	magnesium
mL	milliliter
MQ-water	Milli-Q water
MSM	mineral salt solution
MT	moderately thermophile
mV	millivolt
N_2	nitrogen
NaCl	sodium chloride
NH_4^{+}	ammonium
NO_3	nitrate
OH^{-}	hydroxide ion
ORP	oxidation reduction potential
PCR-DGGE	denaturing gradient gel electrophoresis
PLS	pregnant leach solution
PMF	proton motive force
P_{O2}	oxygen partial pressure
PO_4^{3-}	phosphate
PR	pressure reactor
RO	reverse osmosis
Rpm	rounds per minute
SCE	standard calomel electrode
T	temperature
t	time
TES	trace element solution
V	volt
v/v	volume/volume
Zn^{2+}	zink ion
ºC	Celsius
ΔpH	pH gradient

1. INTRODUCTION

The world´s population is projected to reach 9.8 billion by 2050, which means an approximate 83 million increase yearly (United Nations, 2017). At the same time with the increasing population, the demand for resources will grow. Rapid urbanization is taking place especially in developing countries that results increasing demand for metals by construction industry. (BMI Reserach, 2017). Besides the increasing population, new technologies will also arise that requires high quantity of metals. Estimations says 140 billion tons of yearly minerals, fossil fuels and ores demand by the year 2050, which would be tree times higher than the current consumption. How to meet the demand is one of the big questions of our times. (Lottermoser, 2017). Recycling of metals is getting more attention although as itself it is not a solution for the fulfillment of the metal demand. Majority of the minerals are fixed in buildings which cannot be recycled in the nearest future. (Tilton et al, 2018).

Although new mineral resources in the world are still discovered, their rate is decreasing and are concentrated to certain regions like Africa, China and Southeast Asia (Schodde, 2010). The most significant recent mining activities are taking place in Australia, Canada, Latin America and Africa (Figure 1, Statista, 2018). In many regions of Europe, the mineral resources have been depleted up to depth of 1 km as a result of previous mining activities (Promine, 2018). At these depths, the recovery is not profitable by conventional mining techniques, so the demand of many metals is mostly fulfilled by import. As an example, the European Union´s demand for industry metals (e.g. copper, zink, aluminium) is 20-35% of the global supply and it can fulfill only 3% of the demand by itself. (Matthies et al., 2017; European Commission, 2018). Since near 30 million people are employed in the EU by mineral dependent industries (e.g. automobile, construction, chemical industry, aviation), the dependency of mineral import need to be reduced (Matthies et al., 2017).

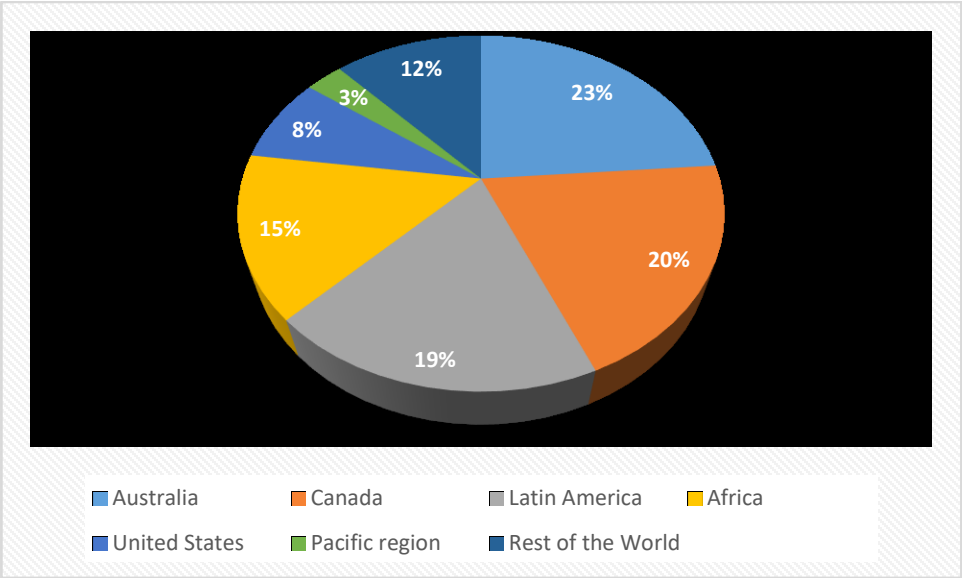


Figure 1: Worldwide distribution of active mineral exploration sites in 2015 by region. Modified from Statista (2018).

Improving the exploration and mining operations is necessary. However, it is not enough simply to improve the extraction efficiency, the solutions need to consider environmental issues and need to be socially acceptable. The mines´ energy use, CO_2 emission, noise pollution and environmental footprint should be decreased. Furthermore, the mines of the future should be less visible for the public than now. To cope with these issues, new innovations and approaches are under research and development. (Lottermoser, 2017). As an example, microbiologically catalyzed *in-situ* leaching (bioleaching) of low-grade ores is potential future approach for extraction of metals form especially deep-buried ores. This approach is currently investigated on a low-grade copper deposit at 1 km depth in a European Commission funded H2020 project BIOMOre. (Matthies et al., 2017).

The aim of this work was to study the effects of elevated pressures on iron oxidation activity by acidophiles (*Leptospirillum ferriphilum, Sulfobacillus sp.*). Hydrostatic pressure increases with depth from the land surface that can influence the biotic iron oxidation (Davidson et al., 1981). Relatively little study has been done about the effects of pressure respect to acidophilic microorganisms. Testing the effect of elevated pressures on iron oxidation activity would give useful information for the future´s deep *in situ* bioleaching. Furthermore, this study was conducted to produce activated carbon-bound iron oxidizing biomass for pilot-scale deep *in situ* application. Using different type and scale biomass production means gives a good view on the limitations of each systems and the overall challenges of biomass scale-up.

2. *IN SITU* BIOLEACHING

Bioleaching is the term used for the solubilization of minerals from ores through biological processes (Kelly et al., 1979). Sometimes mistakenly biooxidation can be used as substitutive term for bioleaching. Although bioleaching includes oxidative processes, but it is not the same as biooxidation because the latter only includes the microbial decomposition of minerals but not the solubilization of gold. (Rawlings, 2002). During bioleaching, the wanted metal is leached into the aqueous solution and then it can be recovered. With the biooxidation only the mineral containing the wanted metal is removed from the ore. (Johnson, 2014). For the extraction of gold from the pretreated mineral after biooxidation, cyanide leaching, or other subsequent chemical leaching step is required to solubilize the gold. Bioleaching and biooxidation belong to the comprehensive term biomining, which includes all technologies that use biological systems to promote metal extraction and recovery. (Rawlings, 2002; Johnson, 2014).

Shortly after life began on Earth, microorganisms that are able to decompose minerals have also evolved. In Roman (first century BC) and probably already in Phoenician times biological activity of microorganisms in leaching of copper and silver has already been utilized without knowing it. (Rawlings, 2002; Brierley, 1982). The Rio Tinto mine in Spain dates back to those ancient times and it was rediscovered in 1556 by Francisco de Mendoza. In those times it was recognized that iron dissolves and later copper precipitates in the Rio Tinto river but the phenomena behind was not understood yet. (Rawlings, 2002). Leaching of mineral resources has become more common in the 18th and early 1920`s in Europe and USA, respectively (Davidson et al., 1981). Although the early leaching practices, the involvement of iron- and sulfur-oxidizing microorganism had not been known until the late 1940´s (Davidson et al., 1981). During the last two decades, understanding the role and the ways to utilize these microorganisms has been developing rapidly (Vera et al., 2013). Nowadays, small percentage of cobalt and nickel, approximately 5% of gold and >15% of copper is recovered by using biomining techniques (dump-, heap and stirred tank bioleaching) (Brierley & Brierley, 2013).

Biomining of low-grade ores is more economically viable than the traditional recovery processes such as leaching of gold and silver ore in cyanide that is followed by solid-liquid separation, washing the solid residues and finally zinc cementation of the leach liquor (Rawlings, et al., 2003; Fleming, 1992). It enables the recovery of metals from low grade ores and even the utilization of waste dumps from previous mining activities is possible. Biomining also creates less chemically active tailings which reduces the risk of unwanted metal pollution and acid created by the mine tailings and wastes. This

biological approach creates own heat and often does not require additional, which makes it much more energy efficient than smelting and roasting. (Rawlings, et al., 2003; Olson et al., 2003). Finally, it helps to cut back the harmful gas emissions (e.g. sulfur dioxide) of traditional mining activities (Rawlings, et al., 2003) and fixes carbon dioxide (Nagpal et al., 1993). The main concerns about the biomining are its long extraction time, need for large metal-extraction reactors in case of using stirred tank bioleaching, reliance on grinding and blasting, need for acid and water pollution control and costs (Brierley, 2008;Johnson, 2018; Gray, 1997).

Although biomining enables recovery of cobalt, zinc, nickel and uranium; it is mostly used for copper leaching and oxidative pretreatment of refractory gold ores (Johnson, 2014; Vera et al., 2013). Recently innovative approaches (e.g. using neutrophilic heterotrophic fungi and bacteria like *Acidothiobacillus spp*.) for the recovery of electronic waste (e-waste) are under development. As an example, printed circuits (e.g. found in computers) are outstanding source of precious metals. (Johnson, 2014). Another recent development specific to $CuFeS_2$ is to use less positive redox potential and temperature during bioleaching with the help of controlled airflow rates. As an example, this approach improves (+33%) the recovery rate of copper. (Third et al., 2002 and Cordoba et al., 2008). Third et al. (2002) used "potentiostat" bioreactor which was designed to discontinue the aeration in the reactor once the redox potential goes above a certain level which was in their case 380 mV (Ag/AgCl). Using this approach resulted in 52-60% recovery efficiency of copper from chalcopyrite which was nearly double as much as was obtained with the continuously aerated reactor (33%) (Third et al., 2002). Besides the recent approaches it is important to mention the bioreductive dissolution of minerals which has high potential for extraction of target meals. Using bacteria to catalyze the reductive processes and the operation under anoxic conditions makes this practice different from current biomining approaches. (Johnson, 2014).

2.1 Bioleaching techniques

There are two main types of bioleaching techniques. One is the irrigation-type and the other one is the stirred-tank type. The irrigation-type techniques include the dump-, heap, heap reactor- and *in situ* bioleaching. (Rawlings, 2002). The first three are based upon the irrigation of the crushed ore in heaps, dumps or columns with leaching solution that first percolates the pile and then leaves as pregnant leaching solution (solution containing the target metals) that is collected for further processing. The ore piles can be even 350 m high in the dump bioleaching (Figure 2 a) while with the heap leaching (Figure 2 b) the piles are only 2-10 m high. This size difference is because the dump bioleaching uses run-

off-ore that is piled up while the heap leaching uses crushed ore that is acidified with sulfuric acid and agglomerated before piling up. Although both bioleaching processes can last some years, the heap bioleaching is more efficient. Both of these two techniques use the naturally occurring microorganisms at the leaching site. (Schnell, 1997). The heap reactor bioleaching is very efficient (e.g. enables the recovery of 1 g Au/tonne ore) but because of its high costs, it is mainly used for the recovery of gold (Whitelock, 1997). While the heaps and dumps are irrigated with leaching solution containing raffinate, iron and recycled wastewater, at the heap reactor the heap is irrigated with acidic ferric iron rich solution that also contains acidophilic bacteria and then with recycled reactor effluent. The metals remain in the heap with this latter technique, so the heap need to be washed to remove cyanide and acid at first and then taken up, reagglomerated with lime, packed in lined pads and finally the metals chemically extracted (e.g. with dilute solution of cyanide). (Schnell, 1997; Rawlings, 2002; du Plessis et al., 2007). The *in situ* bioleaching is based on the same phenomena as the dump- and heap- bioleaching but with this technique the leaching happens at the natural occurrence of the metal containing ore, in this technique, the leaching solution is injected to the subsurface ore body and percolates through natural pathways like crack and voids (results of fracturing). Finally, the pregnant solution is collected through deep drill-holes and pumped to the surface for further processing. (Filippov et al., 2017).

The other main bioleaching technique is based on the use of stirred tanks (Figure 2 c), which enable controlled environment, high aeration and good stirring which makes them more expensive to construct and operate. Stirred tank systems are typically operated in continuous-flow mode and consists of series of bioreactors which are arranged parallel to avoid the wash out microbial cells. (Rawlings, 2002; du Plessis et al., 2007). This stirred tank system has high construction and operational costs, so it is mostly used with high-value ores and concentrates (Lindström et al., 1992; Van Aswegen et al., 1991). They usually enable the complete biooxidation of the mineral concentrate (Rawlings, 2002; du Plessis et al., 2007).

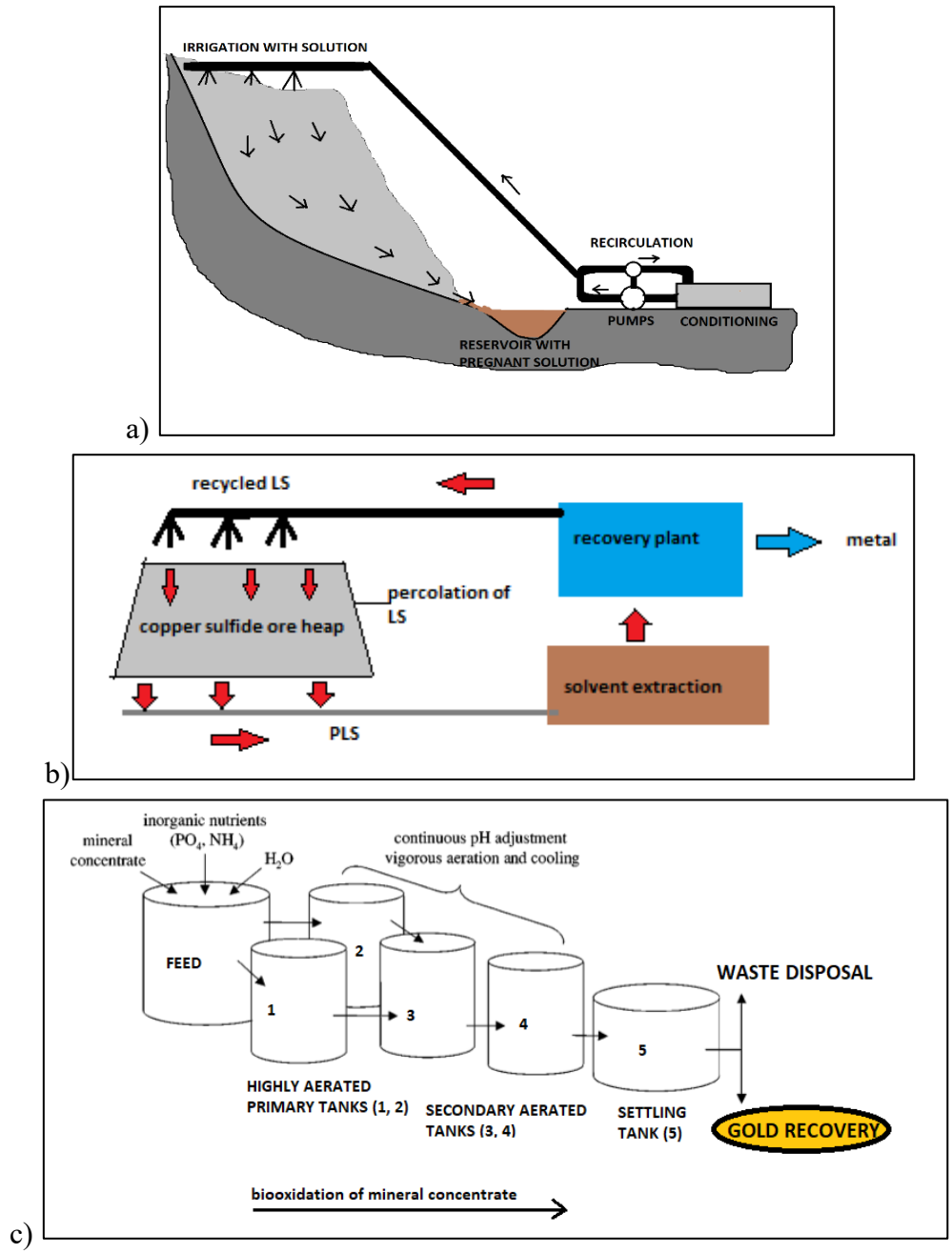


Figure 2: Principles of the most typically used bioleaching techniques: (a) dump bioleaching (modified from Näveke, 1986), (b) heap bioleaching (modified from Rawlings, 2002) and (c) stirred tank bioleaching (modified from Rawlings, 2002).

2.2 Bioleaching mechanisms

From the sulfidic minerals the metals can oxidize to soluble metal sulfates through direct or indirect mechanism. The direct mechanism refers to enzymatic oxidation of the sulfur

from the sulfide mineral, which has been not experimentally demonstrated and thus probably does not exist. (Vera et al, 2013). The indirect mechanism can be divided into contact-, non-contact and cooperative leaching (see Figure 3) (Rawlings, 2002; Rohwerder et al., 2003). Throughout the contact leaching, acidophiles attach to a mineral surface, oxidize sulfide phases and discharge sulfuric acid. At the interface between the sulfide mineral and acidophilic cell wall, electrochemical reaction takes place between the metal sulfide and ferric iron. This reaction then results dissolution of the metal sulfide. (Rohwerder and Sand, 2007). This case there is an interface (layer of extracellular polymeric substances) between the sulfide mineral and bacterial cell. During the non-contact leaching, the ferrous iron (Fe^{2+}) is biologically oxidized to ferric iron (Fe^{3+}) by planktonic cells. The Fe^{3+} together with protons oxidize the metal sulfides. In the cooperative leaching sulfur intermediates, sulfur colloids and mineral fragments are released by the planktonic cells attached to the mineral surface. These released substances then serve as substrates for iron- and sulfur oxidizing microorganisms. (Rawlings, 2002; Rohwerder and Sand, 2007; Rohwerder et al., 2003).

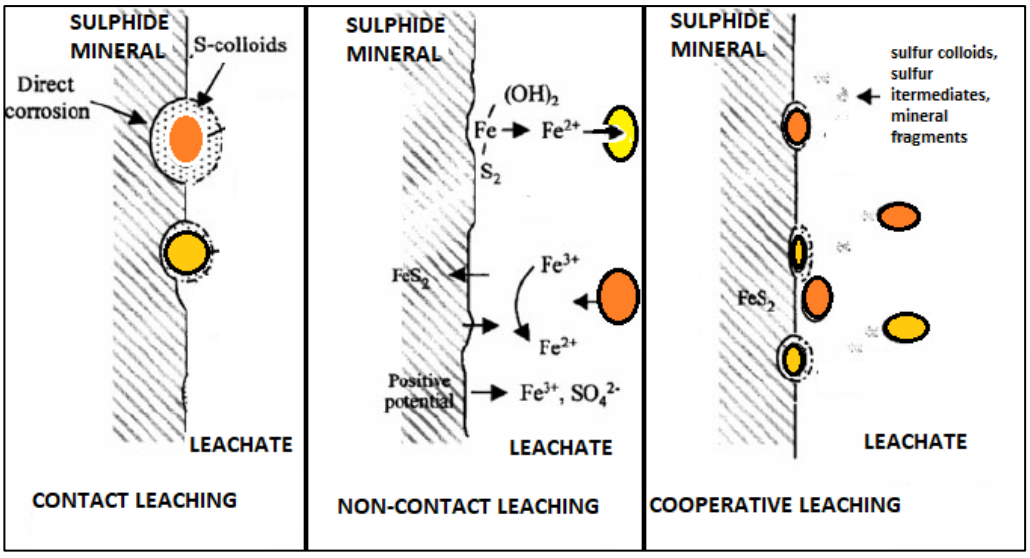


Figure 3: Indirect bioleaching mechanisms. From left to right: contact-, non-contact- and cooperative leaching (modified from Rawlings et al., 1999)

The direct mechanism is summarized in Equation 1 and the indirect one in Equations 2-4 (Bosecker, 1997; Sand et al., 2001; Rohwerder and Sand, 2007 and Rohwerder et al., 2003).

$$MS + 2O_2 \rightarrow M^{2+} + SO_4^{2-} \qquad (1)$$

$$MS + 2Fe^{3+} \rightarrow M^{2+} + S^0 + 2Fe^{2+} \quad (2)$$

$$2Fe^{2+} + 0.5O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O \quad (3)$$

$$S^0 + 1.5O_2 + H_2O \rightarrow 2H^+ + SO2_4^{2-} \quad (4)$$

2.3 Applications of *in situ* leaching

In situ leaching (ISL) that does not rely on activity of any microorganisms (Figure 4), has been applied for the last 67 years. For the recovery of uranium, ISL has been developed in former Soviet Union, Uzbekistan and U.S.A within the 1950´s and 1960´s. (Boytsov, 2014 and World Nuclear Association, 2015). The recovery of uranium from previously used deep mines has been extensively applied in Canada in the 1970´s. The technique used there slightly differed from the recent meaning of ISL. The ore body was fractured by using explosives which was followed by flooding the mines and pumping up the pregnant leach solution (PLS) to the surface for the extraction of uranium. This Canadian application has been considered successful because the uranium recovery just from the Dension mine was approximately 300 tons. (Rawlings, 2002; McCready and Gould, 1990). In the 1980´s, new ISL mines were opened in Czechoslovakia, Bulgaria and China. Besides some small ISL projects in Russia and Australia, the 1990´s was a stagnation period. Since 2000, the ISL of uranium has been booming again. In the 2000´s and 2010´s, new ISL mines were opened in U.S.A., Russia, Uzbekistan and eight new mines in Kazakhstan. In year 2015, 51% of the world´s uranium production originated from mines utilizing ISL. (Boytsov, 2014; World Nuclear Association, 2015).

Besides for the recovery of uranium, ISL has been also used for the recovery of copper for example at San Manuel, Arizona. The technique used consisted of the injection of acidified leaching solution through arrays of wells, collection of gravitated PLS and copper recovery from the PLS at the surface. Because of unsuitable geology of the mining for ISL, 13.5% fluid loss at the mine site was recorded. (Schnell, 1997). Another ISL copper mine is the Mammoth mine in Queensland, Australia (Rawlings, 2002). ISL has been also tested on porphyry copper deposits in USA and applied for the recovery of gold in Russia (Seredkin et al., 2016). In the 1990´s, a combined method for the leaching of gold from gold-bearing regolith was used in the Ural Mountains region. First, the leaching solution was let to infiltrate into the waterless zone above the water table and then the PLS was collected from the top of the water table. Secondly, ISL below the water table also took place (conventional ISL filtration). The gold extraction with this combined method reached 70% recovery efficiency. (Zabolotsky et al., 2008).

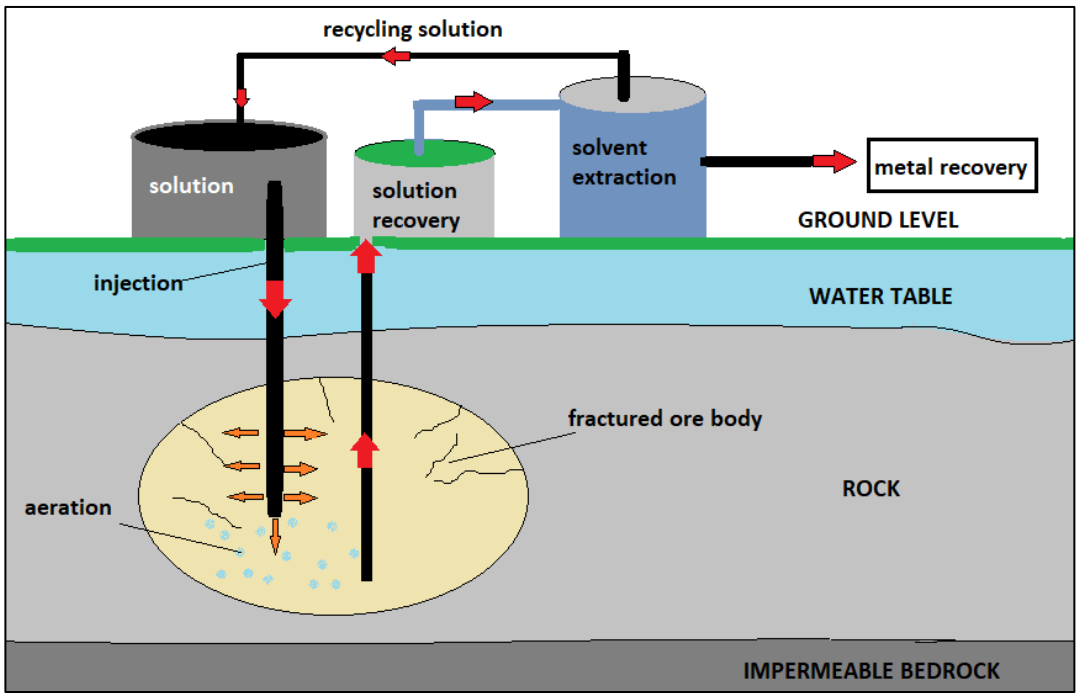


Figure 4: Principle of *in situ* leaching (modified from Davidson et al., 1981)

Since 2015, a novel deep *in situ* mining approach is currently being studied and developed in the BIOMOre project that is funded by the European Commission´s Horizon 2020 research and innovation program and that involves 23 partners from 9 different countries. The main target of the project is to develop an environmentally friendlier and more cost-efficient approach than traditional mining techniques that can be used with low-grade ores and deep-buried ore bodies. The main concept is to couple the deep ISL and indirect bioleaching for the recovery of metals. (Filippov et al., 2017). The uniqueness of this approach is based on the biological regeneration of ferric iron from the ferrous iron of the metal-enriched pregnant liquors that would be recirculated into the ore body. The future concept would be to conduct the leaching underground and the biooxidation of ferrous iron would take place in a ferric iron-generating bioreactor (FIGB) that is located at the land surface. (Pakostova, 2017 and Filippov et al., 2017). Placing the bioreactor on the surface would prevent the negative effects of pressure and low oxygen concentrations that exist deep underground. The main concerns of this approach are the use of hydraulic fracturing and the introduction of bacteria to the subsurface. As a part of the BIOMOre project, the concept is tested in a pilot-scale at a geologically suitable (sandstone) copper mining site at Rudna mine in Poland, where the mine is operated by KGHM Polska Miedz SA. The Kupferschiefer sedimentary copper rich-ore of this mining site is calcareous and contains high amount of halite (NaCl) that is unwanted in bioleaching operations because

in high concentrations, it is inhibitory for acidophilic iron oxidizing bacteria. (Filippov et al., 2017).

Besides the pilot application, the effect of NaCl, pressure and temperature on the microorganisms and on copper recovery kinetics has been also studied at laboratory-scale (Filippov et al., 2017). Pakostova et al. (2017) studied the impacts of elevated chloride concentrations on the activity of iron oxidizing microorganism. They demonstrated that using a combination of water- and acid-leaching prior to ferric iron leaching successfully reduces the negative impacts of the carbonates and chloride present in the ore body to acceptable levels for the acidophiles. (Pakostova et al., 2017). First the halite is removed from the ore by water-washing through the wells. After the washing step, the removal of carbonates by acid washing is done. Once most of the carbonates are removed, ferric iron solution can be injected to the wells for the oxidation of the sulfide minerals and solubilization of copper. After finishing of leaching operation, the pH at the ore is increased close to neutral to prevent the possible bacterial activity. This is the protocol that will be also used at Rudna mine. (Filippov et al., 2017).

2.4 Acidophilic microorganisms used for bioleaching

The microorganisms used during bioleaching operations are bacteria and archaea. All bioleaching microorganisms share common physiological features. First of all, they are all chemolithotrophs, which means that they are able to derive their energy from inorganic reduced compounds. These microorganisms can use ferrous iron and/or inorganic sulfur sources as electron donor and most require oxygen (some can use ferric iron) as electron acceptor. (Dopson et al., 2002). They require carbon-dioxide (CO_2) for their growth that they can fix from the atmosphere. To fulfill the oxygen and CO_2 requirement of these microorganisms, aerated environment should be provided. All bioleaching microorganisms are acidophiles and generally prefer pH levels between 1.4-1.6. These microorganisms can resist a range of metal ions, which makes them suitable for bioleaching applications. (Dopson et al., 2002 and 2003).

The microbial decomposition of the minerals can take place at different temperatures. The acidophiles used with bioleaching operations can be mesophilic (optimum 20-40°C), moderately thermophilic (optimum 40-60°C) and thermophilic (optimum >60°C). Although it is not common but some bioleaching microorganism like some strains of *Acidithiobacillus (A.) ferrivorans* tolerate low temperatures. (Johnson, 2014). Some of the most studied acidophilic microorganisms that are used in bioleaching operations are shown in Table 1. From the bioleaching microorganisms one of the most important acidophile in biomining processes is *A. ferrooxidans*. This bacterium is commonly the

dominating one in heap- and dump bioleaching operations. (for a review, see Rawlings et al., 2002 and 1999). Once there is control of pH and temperature (typically 40-45°C), as is the case in stirred tank systems, the iron oxidizing *Leptospirillum (L.) ferriphilum* or the sulfur-oxidizing *A. caldus* typically become the dominant organisms. The dominating specie in this kind of controlled environment depends on whether iron or sulfur is available in higher quantity the solution. (Okibe et al., 2003; Okibe and Johnson, 2004).

Table 1: p. 1/2 Characteristics of acidophilic bacteria and archaea used in bioleaching operations

Species	Oxidation of iron/sulfur		Temperature (°C)		pH		Type of bioleaching operation	Note	References
Bacteria		**M/ MT/ T/ C**[1]	**range**	**optimum**	**range**	**optimum**			
Acidithiobacillus (A.) ferrooxidans	iron/sulfur	M	10-37	30-35	1.3-6.0	1.8-2.5	stirred tanks	can reduce Fe3+	a, b, c, d, e
A. ferridurans	iron/sulfur	M							a,
A. ferrivorans	iron/sulfur	M,C							a,
A. caldus	sulfur	MT	32-52	45	1.0-3.5	2.0-2.5	stirred tanks		a, b, d, e, f
A. thiooxidans	sulfur	M	10-37	28-30	0.5-6.0	2.0-3.5	heap leaching, stirred tanks		a, b, c, d
Acidiferrobacter thiooxydans	iron/sulfur	M/MT						can reduce Fe3+	a,
Leptospirillum (L.) ferriphilum	iron	MT	<45	30-37		1.3-1.8	stirred tanks	dominant autotroph in stirred tank	a, e, o,
L. ferrooxidans	iron	M	2-37	28-30	0.5->3.5	2.0	heap leaching		a, b, d, e, g, o
Sulfobacillus (Sb.) thermosulfidooxidans	iron/sulfur	MT	20-60	45-48	1.5-5.5	2		can reduce Fe3+	a, b, d, e
Sb. benefaciens		MT					stirred tanks		a,
Sb. thermotolerans	iron and sulfur	MT							a,
Alicyclobacillus spp.	iron and sulfur	MT							a,
Acidiphilium spp.		M						reduce Fe3+, mainly obligate heterotrophs	a,
Acidimicrobium ferrooxidans	iron	MT	<30-55	45-50		2			a, b, e, j,

Table 1: p. 2\2 Continued

Species	Oxidation of iron/sulfur	Temperature (°C)			pH		Type of bioleaching operation	Note	References
		M/ MT/ T/ C[1]	range	optimum	range	optimum			
Ferrimicrobium acidiphilum	iron	M							a,
Archaea									
Ferroplasma acidiphilum	iron	M/MT	15-45	35	1.3-2.2	1.7	stirred tanks	heterotrophic	a, b
Acidiplasma cupricumulans	iron	MT					stirred tanks	heterotrophic	a,
Sulfolobus (S.) metallicus	iron/sulfur	T	50-75	65	1.0-4.5	2.0-3.0	heap leaching	autotrophic	a, e, k, n
S. shibatae-like	sulfur	T						Facultative chemolithotroph	a,
Metallosphaera sedula		T	50-80	75	1.0-4.5	2-3	heap leaching		a, b, d, e, m, n
Acidianus (Ac.) brierleyi	sulfur/iron	T	45-75	70	1-6	1.5-2.0			a, b, d, e, m
Ac. sulfidivorans		T	83-						a,
Ac. infernus	iron/sulfur	T	65-96	90	1.0-5.5	2.0			a, b, d, e, m
Stygiolobus azoricus-like		T						Obligate anaerobe; grows by S- respiration	a,

*Sources: (a) Johnson (2014), (b) Brandl (2001), (c) Krebs et al. (1997), (d) Rawlings (2002), (e) Schippers (2007), (f) Watling (2006), (g) Baker and Banfield (2003), (h) Kinnunen and Puhakka (2005) , (i) Nurmi (2009), (j) Clark and Norris (1996), (k) Golyshina et al. (2000), (l) Huber and Stetter (2001a), (m) Huber and Stetter (2001b), (n) Rawlings (2005), (o) Karavaiko et al. (2006)

[1]M: mesophile; MT: moderately thermophile; T: thermophile; C: cold tolerant

3. CHEMICAL AND BIOLOGICAL IRON OXIDATION

3.1 Process of iron oxidation

The metal sulfide oxidation can follow two chemical pathways (see Figure 5). One is the thiosulfate mechanism, which occurs with acid non-soluble metal sulfides (e.g. FeS_2, MoS_2, WS_2) and the other is the polysulfide mechanism for the acid-soluble metal sulfides (most of the metal sulfides). The ferrous iron oxidation to ferric iron has crucial role during both metal sulfide oxidation pathways. This iron oxidation can be abiotic or biologically catalized. (Rohwerder and Sand, 2007; Schippers and Sand, 1999). In natural environments the abiotic and biotic iron oxidation is inseparable (Ionescu et al., 2014).

There are also two pathways the abiotic Fe^{2+} oxidation can follow. The first is the homogenous pathway which occurs in solutions and the other one is the heterogenous one which is in association with mineral surfaces. (Jones et al., 2015 and Theis et al., 1974). Throughout the heterogenous pathway, mineral surfaces help to catalyze the iron(II) oxidation and at the same time, drive the formation of crystalline Fe^{3+} -oxides (Chen and Thomson, 2018). The iron oxidation can be catalyzed by acidophilic iron oxidizing microorganisms. In case of the thiosulfate pathway, the Fe^{3+} oxidizes the metal sulfides via electron extraction. The Fe^{3+} has the same role during the polysulfide pathway but this also requires proton attack for the oxidation of metal sulfides. (Rohwerder and Sand, 2007; Schippers and Sand, 1999).

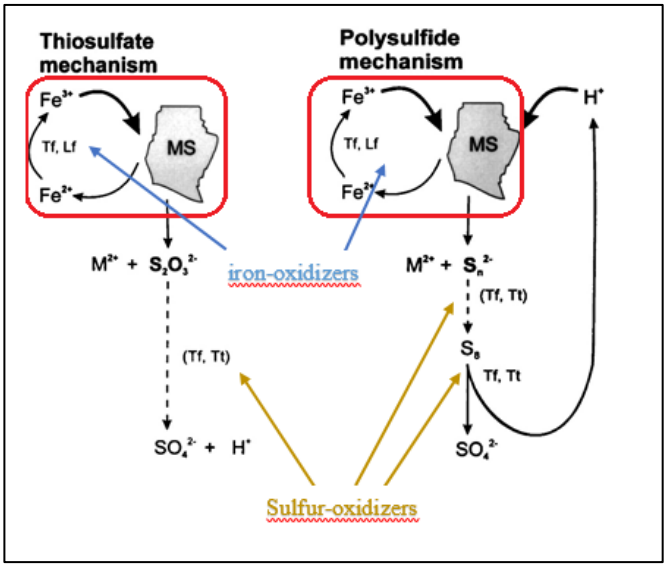


Figure 5: Thiosulfate and polysulfide oxidation pathways (modified from Rohwerder and Sand, 2007). The iron oxidation and the part of electron extraction by Fe^{3+} are shown inside the red rectangle.

The stoichiometry of ferrous iron oxidation is shown in the Equation 5 (Sand et al., 1995). This reaction can be catalyzed by acidophilic iron oxidizing microorganisms (Schipper and Sand, 1999).

(5)

$$2Fe^{2+} + 0.5O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O$$

The abiotic ferrous iron oxidation in homogenous solutions with pH> 5 mainly depends on the partial pressure of oxygen (pO_2/ atm) and the OH^- activity (OH^-/M) (Haber and Weiss, 1934). This oxidation can be described by the Haber-Weiss mechanism sown in the Equation 6 (Stumm and Lee, 1961).

(6)

$$\frac{d(Fe(OH)_3)}{dt} = k \cdot (Fe^{2+}) \cdot p(O_2) \cdot (OH^-)^2$$

In the Equation 6 the *k* is the reaction rate constant in L^2/mol^2 atm min, the (Fe^{2+}) is the ferrous iron concentration in the solution in mol/L, $p(O_2)$ is the partial pressure of oxygen in atm and the (OH^-) is the hydroxide concentration in mol/L.

Besides the oxygen concentration and pH of the solution many other factors like concentration of ferric iron, copper, manganese, silica; temperature and alkalinity have effect on the rate of oxidation (Stumm and Lee, 1961 and Ghosh et al, 1996).

3.2 Iron oxidizers

Most studied iron oxidizing microorganisms are *A. ferrooxidans* and *L. ferrooxidans*. From the iron oxidizers at least 14 genres can utilize molecular oxygen as electron acceptor during ferrous iron oxidation. (Bonnefoy and Holmes, 2011; Blake and Griff, 2012). Some of the iron oxidizers (e.g. L. *ferriphilum, L. ferrooxidans, Acidimicrobium ferrooxidan, Ferrimicrobium acidiphilum*) are only able to oxidize ferrous iron and some (*A. ferrooxidans, A. ferridurans, A. ferrivorans, Sulfobacillus (Sb.) thermosulfidooxidans, S. thermotolerans, Alicyclobacillus spp., S. metallicus, Acidianus (Ac.) brierleyi, A. infernus*) can switch to sulfur oxidation once ferrous iron is absent (see from Table 1) (Johnson, 2014).

4. PARAMETERS AFFECTING IRON OXIDATION

Several physicochemical parameters such as temperature, pressure, dissolved oxygen (DO) concentration, iron, other heavy metals and chloride concentrations, pH and redox potential of the solution, and the availability of nutrients affect significantly the rate and efficiency of iron oxidation. Specific effects of each of these parameters are discussed in the following sections.

4.1 Parameters specific to deep subsurface application

There are location specific parameters like temperature, pressure and dissolved oxygen that need to be considered before deep subsurface iron oxidation. Besides these three, chloride content as fourth parameter need to be also taken into account before iron oxidation to leaching of saline, calcareous copper sulfide ore which is the case with the first BIOMOre application at the Rudna mine in Poland.

4.1.1 Temperature

The abiotic iron oxidation rate increases by temperature. The effect of temperature on the chemical processes can be described by the Arrhenius equatinon (Equation 7). In the Equation 7 the k is a constant at a temperature of interest ($k(T)$) or at a reference temperature ($k(T_{ref})$) in Kelvins (K). The E_a is the activation energy in J, kJ or cal/mole and the R is the universal gas (Regnault) constant. Considering that the T_{ref} is fixed and the $k(T_{ref})$ is a measured quantity, only the E_a controls the iron oxidation rate constant at temperature of interest. The Equation 7 can be simplified to the following Equation 8, where the T and T_{ref} has unit in ºC and the c is a constant in ºC^{-1}. (Peleg et al., 2012).

$$\frac{k(T)}{k(T_{ref})} = Exp\left[\frac{E_a}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] \quad (7)$$

$$\frac{k(T)}{k(T_{ref})} = Exp\left[c\left(T - T_{ref}\right)\right] \quad (8)$$

The biotic iron oxidation behaves differently than the abiotic one with respect to changing temperature. To describe the relationship between the microbial activity and the solutions´ temperature the Ratkowsky´s equation (Equation 9) is commonly used (Ratkowsky et al., 1983).

$$\sqrt{\frac{1}{t}} = b * (T - T_{min}) * (1 - e^{(c*(T-T_{max}))}) \quad (9)$$

In the Equation 9 *t* is the time that is required for the oxidation of half of the initial ferrous iron concentration (e.g. in hours (h)), *b* is a regression coefficient, *T* is the absolute temperature (°C) and *c* is an additional fitting parameter. The T_{min} is the minimum and T_{max} is the maximum temperatures where no cell growth occurs. The *t* required can be calculated from the initial ferrous iron concentration and the zero order rate constant of the temperature. The calculation of *t* can be seen from the Equation 10 where the C_o is the initial Fe^{2+} concentration and k is any temperatures´ zero order rate constant (K). (Ratkowsky et al., 1983).

$$t = \frac{Co}{2 \cdot k} \quad (10)$$

The biotic iron oxidation can take place within 0-85°C. From the acidophilic microorganisms responsible for the ferrous iron oxidation, psychrophiles can grow at temperatures 0-25°C (optimum typically close to 15°C), mesophiles at 15-45°C (optimum typically 25-35°C), moderate thermophiles at 40-60°C, thermophiles at 60-80°C and hyperthermophiles at above 80°C. (Kaksonen et al., 2008; Plumb et al., 2007b; Ahonen and Tuovinen, 1989).

The dominating iron oxidizing microorganisms at mesophilic conditions in stirred tank system are *Acidithiobacillus ferrooxidans* (formerly *Thiobacillus ferrooxidans*) and *L. ferrooxidans* and within 40-60°C *A. caldus and Sulfobacillus (Sb.) thermosulfidooxidans* (Kelly and Wood, 2006 and Brandl, 2001). As an example, one of the most commonly studied iron and sulfur oxidizing mesophile *A. ferrooxidans* has its optimum growth temperature at 25-35°C. It has been demonstrated that iron oxidation can even occur at

temperatures as low as 5-6°C. (Ferroni et al., 1986). The decrease of temperature can elongate the lag-phase of iron oxidation. Dopson et al., (2006) reported that 2.5-fold decrease in temperature (21.8 to 8.6°C) resulted in 3.3-fold longer lag-phase of iron oxidation by *A. ferrooxidans* (Watling et al., 2016). The rates of chemical reactions during iron oxidation can be doubled by increasing the temperature with 10°C (for a review, see Rawlings et al., 2003).

4.1.2 Pressure

Pressure oxidation can be used to break down the iron sulfide mineral which enables the recovery of the wanted metal (e.g. refractory gold concentrate) (Fleming, 2009). During the pressure oxidation, the iron sulfide mineral oxidation is initiated by the pressurized steam. This process releases heat that sustains the reaction. The principal oxidant is oxygen of the pressure oxidation process. (U.S. EPA, 1994). During the process, sulfuric acid is generated that facilitates the release of the precious metal from the sulfide crystal which makes further recovery (e.g. by cyanide leaching) possible. At the same time iron goes into the solution in the form of ferrous sulfate which is quickly oxidizes to ferric sulfate. Finally, the ferric sulfate hydrolyzes and reprecipitates to hematite, iron sulfate or jarosite. Typically, autoclave is used for the pressurization during pressure oxidation. Generally, the oxygen pressure of the process is 3.5 to 7 bar and the temperature is 190°C to 230°C respectively. (Fleming, 2009).

The increase of hydrostatic pressure of a water column by depth is approximately 10.1 bar per every 10 m, so in the case that biological iron(II) oxidation would be applied deep underground, discovering pressure tolerant acidophilic microorganisms is essential (ZoBell and Hittle, 1967; Davidson et al., 1981). Studies like the one made by Davidson et al. (1981) have shown that under anaerobic conditions the application of elevated pressure has only minor effect on microbial growth. They reported that elevated hydrostatic pressures as high as 304 and 253 bar did not prevent the growth of *T. ferrooxidans* and TH3 (*Thiobacillus* like bacterium) in Pyrex tubes, respectively. In this growth under pressure experiment, the oxygen and carbon-dioxide were provided with air-saturated fluorocarbon to the media. Only minor effect on iron oxidation by *T. ferrooxidans* was reported during the application of hydraulic compression up to 689 bar. Although the resistance to high pressure, it was recognized that the previously pressure treated cells consumed 38% less oxygen than the control culture that was continuously kept at atmospheric pressure. Also, the ability of these decompressed cells to incorporate carbon dioxide was mostly lost. This same study has shown that changing the gas of pressurization from helium (He) to air, has shown inhibitory effect already at 1 bar. At 10

bar sterilization and at 8.7 bar only minimal growth occurred of the TH3 culture. (Davidson et al. 1981). More recent study by Zhang et al. (2017) showed high pressure tolerance (up to 100 bar) of the iron(III) reducing biomining culture (*Acidianus brierleyi, Thermoplasma acidophilum and Sulfolobus metallicus*) under anaerobic conditions. ZoeBell et al. (1967) and Fenn and Marquis (1968) reported according to Davidson et al. (1981) that 10.1-50.7 bar inhibits the multiplication of most of the aerobic bacteria. Davidson et al. (1981) documented negative effect of compressed air on biological iron oxidation, while it was improving the chemical oxidation.

4.1.3 Dissolved oxygen

Whether iron oxidation is abiotic or biotic, depends on the oxygen (O_2) concentration of a solution (Morgan and Lahav, 2007). The overall demand of oxygen in the liquid phase depends on diverse chemical and microbial oxidation reactions. To maintain high efficiency, the oxygen transfer rate from the gas-phase should exceed or at least equal the demand within the liquid-phase. (du Plessis, 2007). At circumneutral pH with low O_2 concentration, the rate of abiotic and biotic Fe^{2+} oxidation is very similar while with high O_2 concentration, the abiotic one dominates (Emerson et al., 2010 and Druschel et al., 2008). The study by Chen and Thomson (2018) showed that the iron(II) oxidation efficiency decreases by the reduction of partial pressure of O_2 (pO_2). Increasing the pO_2 from 1% to 21% with the same initial iron(II) concentration, resulted 24 times faster iron (II) oxidation in their study.

Most acidophilic microorganisms in bioleaching operations are aerobic and the most current bioleaching operations rely on oxidative bioleaching. O_2 is essential for the oxidative metabolism of the iron oxidizers, as it is the electron acceptor of ferrous iron oxidation. (Halinen, 2015). As an addition to the oxidative metabolism, the dissolved oxygen is also crucial for the active growth of most of the acidophilic microorganisms (Mohapatra, 2006). The gas mass transfer rate into the liquid is dependent on temperature (Figure 6) (du Plessis et al., 2007). Often the available O_2 is not adequate so it need to be artificially supplied during the bioleaching operations like heaps and reactors (Halinen et al., 2015).

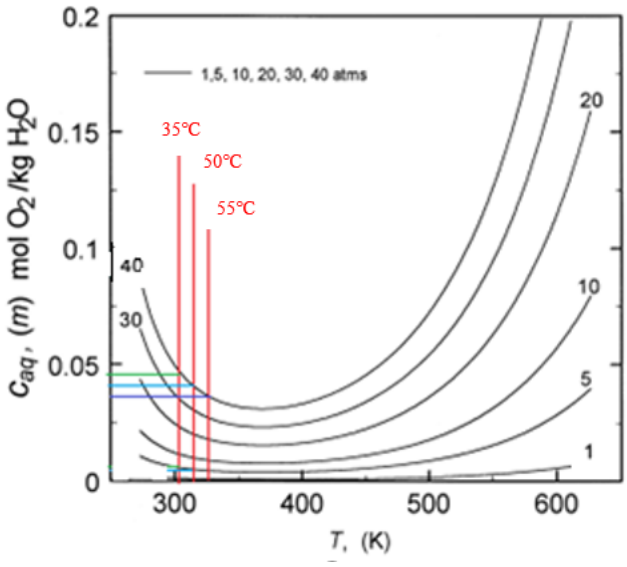


Figure 6: Effect of temperature and partial pressure of oxygen (P_{O2}) on the solubility of oxygen in water (c_{aq}). The temperatures typically used in bioleaching operations in Celsius are marked with red vertical lines, the different pressure (in atm) curves are shown with black and the oxygen solubility at 35°C, 50°C and 55°C are marked with green, blue and purple horizontal lines respectively. The figure is modified from Tromans (1998).

The theoretical dissolved oxygen (DO) concentration at a certain oxygen partial pressure and temperature can be estimated by using a thermodynamic (Equation 11) of Tromans (1998). In the Equation 11, the c_{aq} is DO concentration given in mol/L, P_{O2} is oxygen (O_2) partial pressure in atm, and T is temperature in Kelvin (K).

(11)

$$\mathbf{c_{aq}} = \mathrm{P_{O_2}} \exp\left\{\frac{0.046\mathrm{T}^2 + 203.357\mathrm{T}\ln\left(\frac{\mathrm{T}}{298}\right) - (299.378 + 0.092\mathrm{T})(\mathrm{T} - 298) - 20.591\mathrm{x}10^3}{8.3144\mathrm{T}}\right\}$$

4.1.4 Chloride

Many of the metal rich ore deposits are in arid and semi-arid areas where the available water either has low-quality or the water availability is limited. Mining operations require huge amount of water and this requirement needs to be fulfilled by alternative sources in case lack of clean water. In these regions desalination of seawater or recirculation of

process water are possible options. Seawater has high salinity (~500 mM NaCl) which is inhibitory or at least has negative impact on the metabolic functions of most of the microorganism used during bioleaching operations. (Johnson et al., 2015; Davis-Belmar et al., 2014). Reverse osmosis (RO) is generally used for the desalination of seawater, but RO is highly expensive process that makes it unsuitable for large-scale application like mining. Furthermore, its environmental impacts like noise and air pollution, and reduction of recreational fishing areas are also under concern. (Dawoud and Mulla, 2012; Davis-Belmar, 2014; Tularam & Ilahee, 2007). The recirculation of process water can also be problematic. The process water of mining operations might contain chloride (coming from halite) that was dissolved from the treated ore, which makes its proper cleaning before reusing is essential. (Davis-Belmar, 2014 and Filippov et al., 2017).

Some of the ores can also contain chloride that can be liberated during the bioleaching process. As an example, Pakostova et al., 2017 has studied the Kupferschiefer ore from the Rudna mine in Poland which was containing significant amounts of NaCl. They reported liberation of chloride from the ore during indirect bioleaching. Kinnunen and Puhakka (2004) reported that in elevated temperatures (e.g. 67-87°C) the presence of moderate concentration of chloride ions improves the chalcopyrite leaching by ferric sulfate. Their study showed improvement of the copper yields from chalcopyrite with 60, 80 and 100% with the addition of 0, 1 and 5 g/L Cl^-, respectively.

Although there are some NaCl tolerant iron oxidizing microorganism, most of them cannot tolerate high concentrations of chloride ions. Acidophiles, which are used in current bioleaching operations, have positive internal cell membrane which is permeable to the negatively charged chloride ions. Once the chloride ions enter the cell, negative gradient development of the membrane takes place and enables uptake of ions including protons. The uptake of protons causes disturbance of the cytoplasmic pH, which then turns into acidic from neutral. Neutral pH of the cytoplasm is essential for the maintenance of cellular functions so this acidification results to death of the cell. (Watling et al., 2016; Alexander et al., 1987).

The different microbial species involved in bioleaching have different level of NaCl tolerance (Table 2) and some like the iron/sulfur-oxidizing halotolerant *Thiobacillus prosperus* even require it for the growth and iron oxidation (Nicolle et al., 2009). Although chloride is essential for *Thiobacillus prosperus*, at high concentrations it impacts the cell growth negatively, reduces Fe^{2+} oxidation efficiency, can even inhibit the Fe^{2+} oxidation system and lowers the proton motive force of the other iron oxidizing microorganisms (Gahan et al., 2010 and Carla et al., 2012).

Table 2: Chloride tolerance of selected iron oxidizing microorganisms

Acidophilic microorganism	**Chloride (Cl^-) concentration**	**Effect on iron oxidation and/or cell growth**	**Reference**
Acidothiobacillus (A.) ferrooxidans (previously known as *Thiobacillus prosperus)*	≥ 6.1 g/L	inhibitory to cell growth	Huber and Stetter, 1989; Romero et al., 2003
Leptospirillum ferriphilum	> 20 g/L	> 20 g/L inhibitory to cell growth and iron oxidation ≤ 5 g/L no effect, 10 g/L reduces oxidation efficiency	Kinnunen and Puhakka, 2004; Gahan et al., 2009
Thiobacillus prosperus	18.2 g/L optimal	optimal	Huber and Stetter, 1989
A. thiooxidans	0.5 M (~29.2 g/L)	able to growth	Johnson et al., 2015
Sulfolobus (S.) acidocaldarius	≥ 0.32 M (~18.7 g/L)	inhibitory to cell growth	Grogan, 1989
S. metallicus	> 0.513 M (~30 g/L)	inhibitory to cell growth	Huber and Stetter, 1991
S. shibatae and S. solfataricus	≥ 0.32 M (~18.7 g/L)	inhibitory to cell growth	Grogan, 1989
Acidianus brierleyi	≥ 0.17 M (~10 g/L)	no iron oxidation and inhibitory for cell growth	Serger et al., 1986
Acidianus sulfidivorans	≥ 0.17 M (~10 g/L)	inhibitory to cell growth	Plumb et al., 2007a
Metallosphaera cuprina	>0.17 M (~10 g/L)	inhibitory to cell growth	Liu et al., 2011

4.2 Other parameters affecting bioleaching

4.2.1 Solution pH and redox potential

The abiotic iron oxidation rate is affected by the pH of a solution. At pH values below ~4 and above ~8 the abiotic iron oxidation rate does not change while between pH 5 and 8 it increases by the increase of pH. At different pH levels, the distribution of the soluble iron species Fe^{2+}, $FeOH^{+}$ and $Fe(OH)_2^0$ varies. Below pH ~4, Fe^{2+} dominates in the aqueous solution and above pH 8, $FeOH^{+}$. The increase of pH within 5-8 results in increasing concentration of $Fe(OH)_2^0$ in the solution and at the same time improvement of iron oxidation. This improvement occurs because $Fe(OH)_2^0$ is much more readily oxidized than Fe^{2+} or $FeOH^{+}$. (Barak and Lahav, 2007). The abiotic and biotic rate of iron oxidation at circumneutral pH is nearly the same (Ionescu et al., 2014).

Biotic iron oxidation by acidophilic iron oxidizing microorganisms can take place at pH within 0-3 (Halinen et al., 2009a). Although some archaea like the iron oxidizing *Ferroplasma acidarmanus* can grow at pH 0, growth of most of the mineral oxidizers is inhibited at pH values below 1 (Johnson and Hallberg, 2007). Oxidation of minerals and optimal growth acidophilic iron oxidizers usually happens at pH 1.5-2.0 (Dorado et al., 2012). In this range of pH, the redox potential in the solution is relatively high which is beneficial for the leaching of sulfide minerals (Halinen et al., 2009a).

Although, pH below 3 is preferred by most acidophiles, these organisms require circumneutral intracellular pH. This pH difference across the cytoplasmic membrane (pH gradient, ΔpH) is the main promoter to the proton motive force (PMF). This ΔpH is typically 4-5 for acidophilic microorganisms. Because of the net force across the cell membrane, energy dependent processes can be driven by the cells. (Baker-Austin and Dopson, 2007).

The oxidation of ferrous iron into ferric iron consumes acid so increases the pH of the solution while the hydrolysis of ferric iron produces acid and by this makes more acidic condition. Jarosite precipitation produces acid, so for the regulation of too high pH levels, precipitation of some iron as jarosite can be an option. (Nemati et al., 1998).

In solutions with low pH, the ORP of Fe^{2+}/Fe^{3+} is considerably high (+0.77 V, vs standard hydrogen electrode, (SHE)). In this high ORP, relatively large amount of ferrous iron need to be oxidized for the maintenance of the growth of the iron oxidizers. (Holmes and Bonnefoy, 2007). Besides the pH of a solution, the iron solubility is also affected by the ORP. While the chemical iron oxidation happens generally around 200-400 mV (vs.

saturated calomel electrode (SCE)), the redox potential of bioleaching is >500 mV (vs. SCE). (Ahonen and Tuovinen, 1995). Initially high redox potential causes passivation of chalcopyrite which prevents the further dissolution, thus maintaining the redox potential at low levels can be used to accelerate the dissolution of chalcopyrite (Córdoba et al., 2008; Gericke et al., 2010; Petersen and Dixon, 2006; Third et al., 2002). Spectroscopic biochemical and "omics" analyses showed that the ferrous iron oxidizing system respect to redox component differs by iron oxidizing species (Bonnefoy and Holmes, 2011; Blake and Griff, 2012). ORPs measured with Ag/AgCl and SCE are +0.199V and +0.280V vs. SHE, respectively (UBC Chem-E-Car, 2015).

4.2.2 Concentration of iron, other heavy metals and ions

Some amount of Fe^{3+} enhances the rate of oxidation of Fe^{2+} (Wang and Liu, 2014). The presence of mineral surfaces also help to catalyze the heterogenous iron oxidation by driving the crystalline Fe^{3+}-oxide formation (Chen and Thomson, 2018). The abiotic iron(II) oxidation study done by Chen and Thompson (2018) showed that half-life of Fe^{2+} can be reduced just to couple of minutes with the addition of goethite ([56Fe]Gt) to the solution at the presence of 21% O_2 at atmospheric pressure comparing to 4 hours without the goethite. At 21% O_2, the iron(II) oxidation rate was increased by 19-fold and 3-fold by the addition of Gt and γ-Al2O3, while at 1% O_2 concentration the improvement was 8- and 3-folds, respectively.

The activity of iron oxidizing microorganisms can be inhibited by metal accumulation inside the cell at high metal concentrations (Dopson et al., 2003). Biochemical reactions require trace elements such as iron and manganese. Although ferrous iron is essential for the acidophilic iron oxidizing microorganisms, at too high concentrations it can become inhibitory. Besides the microbial strains used, the inhibitory concentration depends on physiochemical parameters like the pH and temperature of the solution. (Ahoranta et al., 2017a). Ahoranta et al. (2017a) reported no inhibition of a mixed bioleaching culture (*A. ferrooxidans and Acidiphilium, Leptospirillum, Ferrimicrobium* sp.) up to 16 g/L Fe^{2+}, and improvement of oxidation efficiency by the increase of ferrous iron concentration from 5 to 16 g/L of Fe^{2+}. Partial inhibition of *L. ferriphilum* and *A. ferrooxidans* has been shown to occur at 30 g/L initial concentration of Fe^{2+} (Kinnunen and Puhakka, 2005 and Nemati and Harrison, 2000). The Fe^{2+} resistance of *L. ferriphilum* dominated culture was shown to be considerably lower (> 4 g/L) than of the mixed cultures of *L. ferriphilum* and *A. ferrooxidans* (Özkaya et al., 2007).

In general, the increase of ferrous iron concentration improves the efficiency of iron oxidation. Increasing the Fe^{2+} concentration results higher ferric iron concentrations in

the solution. Although of Fe^{3+} in the solution can enhance the homogeneous Fe^{2+} oxidation, at high concentrations precipitation occurs, which results passivation of the wanted minerals. (Buamah et al, 2009; Ahoranta et al., 2017a).

Besides keeping the iron concentration below inhibitory levels, regulating other metal concentrations like mercury (Hg^{2+}), cadmium (Cd^{2+}) and silver (Ag^{+}) is also crucial. Toxic effect of these metal cations highly depends on their oxidation state. (Amonette et al., 2003). Ahoranta et al. (2017a) reported that up to 12 g/L Al^{3+} elongates the lag-phase of iron oxidation but it has no toxic effect on iron oxidizers. They also showed that concentrations ≤ 6 g/L might improve iron oxidation (Ahoranta et al., 2017a). Ojumu et al. (2007) reported negative effect of Al on iron oxidation rates and biomass growth with all tested concentrations (2.2-10 g/L). Cd^{2+} forms toxic complexes which is more tolerated by a diverse microbial community than by *A. ferrooxidans* alone (Cabrera et al., 2005). Baillet et al. (1997) reported tolerance of Cd^{2+} up to 112 g/L, whereas Ahoranta et al. (2017a) had only 0.016 g/L. Ag^{+} inhibits the growth of acidophilic microorganisms already at micro molar concentrations (Johnson et al., 2017). As an addition to the above-mentioned metals, the effect of Zn^{2+}, Ni^{2+} and Mg on acidophilic iron oxidizers has been also tested. Nurmi et al. (2009) observed that *L. ferriphilium* tolerates higher concentration of Ni^{2+} than Zn^{2+}. At initial 4 g/L Fe^{2+}, complete iron oxidation occurred within 70 and 45 days with concentrations of 60 g/L Zn^{2+} 50 g/L Ni^{2+} respectively. During the same study, it was also recognized that once these two metals are used together, the negative effects are lower than when only one of metals is present. In case of using *A. ferrooxidans*, the acceptable concentration of Ni^{2+} varies within 10-60 g/L (Özkaya et al., 2007).

Acidification of the cytoplasm can occur in high SO_4^{2-} levels, which have negative, even inhibitory effect on the microbial activity (Rea et al., 2015). The toxic concentrations of SO_4^{2-} differs by the indigenous microbial strain. *Leptospirillum-like* bacterium is rather tolerant (even 150 g/L) than *A. ferrooxidans* which is inhibited at concentration of 130 g/L. (Ahoranta et al., 2017a).

4.2.3 Nutrient availability

For the sustaining or enhancing the activity of iron oxidizers, wide range of macro- and micronutrients are necessary. In general, sulfidic ores contain the essential trace metals but the macronutrients such as potassium (K) and ammonium (NH_4^{+}) may need to be supplied. (du Plessis et al., 2007) and also phosphate (PO_4^{3-}) occasionally to the bioleaching process solution (Rawlings, 2007). Elements like carbon and nitrogen are crucial constituents of nucleic acids and proteins, and thus required for biomass

generation. Some acidophilic microorganisms (e.g. *A. ferrooxidans*) can fix atmospheric N_2, which indicates that nitrogen does not need to be artificially supplied. (Ahoranta et al., 2017a; Levicán et al., 2008). However, some iron oxidizers (e.g. *L. ferriphilum*) cannot fix N_2, so they should be supplied with NH_4^+ (d´Hugues et al., 2008). The study of Sarcheshmehpour et al. (2009) demonstrated enhancement of iron oxidation by supplying NH_4^+. Ahoranta et al. (2017a) suggested addition of 320 mg/L of nitrogen (NH_4^+) to the heap leaching process liquors for the enhancement of iron oxidation. In contrary to the positive effect of NH_4^+, increased concentrations of NO_3 is disadvantageous for the microbial activity (Harahuc et al., 2000; Sarcheshmehpour, 2009). Besides the previously mentioned macronutrients, Ojumu et al. (2007) demonstrated that magnesium (Mg) at low concentrations ($\leq$ 3.05 g/L) is beneficial for the cell growth but higher concentrations depress the specific rate of iron oxidation.

5. MATERIALS AND METHODS

5.1 Introduction to the conducted experiments and biomass cultivation methods

Experimental part of this study consisted of two separate parts. In the first part, the effect of elevated pressures on iron oxidizing microorganisms was studied by using a pressure reactor. Experiments at each studied pressures were operated separately with and without microbial inoculum and the iron oxidation activity of the inoculated cultures were determined after the pressure experiments in shake flask incubations conducted at atmospheric pressure.

In the second part of this study, activated carbon (AC) bound acidophilic iron oxidizing microbial biofilm was grown in cultivation units with increasing scales. The aim of this part was to produce active iron oxidizing microbial biomass to be used as inoculum for a pilot-scale bioreactor that will be used to demonstrate *in situ* bioleaching approach using Kupferschiefer ore block in Rudna mine, Poland. Different laboratory-scale biomass growth methods (shake flask cultures, different types of stirred tank reactors) were utilized at the same time. All the biomass produced at laboratory-scale was used as inoculum in a semi-pilot reactor and once biomass was produced in the semi-pilot reactor, the produced biomass was transferred to the pilot-reactor.

5.2 Inoculum

In all the biological experiments of this study, a mixed microbial sample originating from a Finnish mine site was used as the inoculum. This microbial culture had been enriched for acidophilic iron and sulfur oxidation as consecutive shake flask incubations using either ferrous iron and elemental sulfur or a low-grade gold ore as electron donors. Prior to this study the culture had been incubated in a continuously fed (feed pH 1.3) fluidized bed reactor system (FBR) for oxidation of soluble ferrous iron for 67 days at 35°C. The FBR-grown culture is from now on referred to as the enrichment culture. Based on polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE) followed by sequencing, the culture was dominated by *Leptospirillum (L.) ferriphilum* and contained some *Sulfobacillus* sp.

The temperature of the enrichment culture was maintained at 35±2°C by using a heating blanked over the FBR and the pH at 1.4-1.7 by using a ferrous iron feed solution with a pH of 1.3. For more details about the FBR, see the chapter 6.4.2 Means of biomass cultivation.

5.3 Experimental procedures for studying the effect of high pressure on the activity of the microorganisms

The study of pressure effect on the mixed acidophilic iron oxidizing culture had different experimental steps. As it can be seen from Figure 7, the inoculum used with the pressure reactor was pre-cultivated in shake flasks (inoculum production shake flasks). To see how active the acidophilic culture was after the pressure experiments, some culture solution from the pressure reactor was transferred to shake flasks with fresh media. These shake flasks for the activity testing will be referred to as the "activity testing shake flasks" from now on.

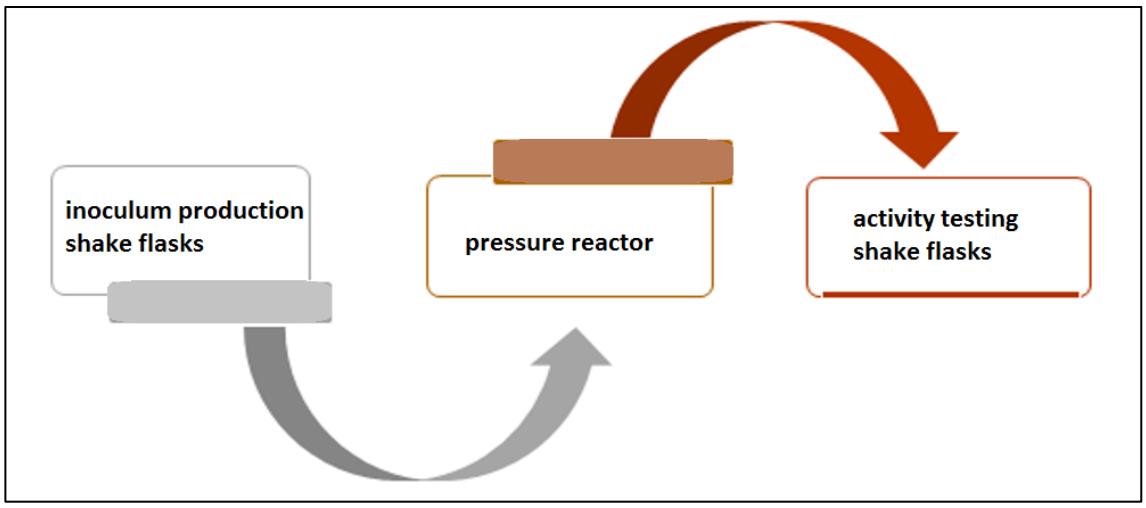


Figure 7: Schematic diagram of the experimental steps used to study the effect of high pressure on the mixed acidophilic culture. The shake flask cultures used as inoculum for the pressure experiments were cultivated at 1 atm, 35°C and 150 rpm for 6 days. The pressure experiments were run with and without inoculum at 35°C and 150 rpm but with different pressure levels for 7-8 days. The culture solution from the pressure reactor was used to inoculate shake flask cultures (activity testing shake flasks), which were incubated at 1 atm, 35°C and 150 rpm for 7 days.

5.3.1 Medium

The culture medium that was used throughout the pressure reactor and shake flask experiments contained 10% (v/v) mineral salts medium (MSM), 1% (v/v) of trace element solution (TES) and ferrous sulfate (with 5.6 or 10.0 g/L Fe^{2+}). The MSM was prepared by adding 37.5 g/L $(NH_4)_2SO_4$, 18.75 g/L $Na_2SO_4 \cdot 10H_2O$, 1.25 g/L KCl, 0.625 g/L K_2HPO_4, 6.25 g/L $MgSO_4 \cdot 7H_2O$, and 0.175 g/L $Ca(NO_3)_2 \cdot 4H_2O$ to Milli-Q water. The pH of the MSM was adjusted to 1.8 with >95% H_2SO_4. The TES contained 1.375 g/L $FeCl_3 \cdot 6H_2O$, 0.0625 g/L $CuSO_4 \cdot 5H_2O$, 0.25 g/L H_3BO_3, 0.319 g/L $MnSO_4 \cdot 4H_2O$, 0.1 g/L $Na_2MoO_4 \cdot 2H_2O$, 0.075 g/L $CoCl_2 \cdot 6H_2O$, 0.1125 g/L $ZnSO_4 \cdot 7H_2O$, and 0.1125 g/L Na_2SeO_4 in Milli-Q water. The pH of the TES was adjusted to 1.5 with concentrated sulfuric acid. Both the MSM and TES were sterilized by autoclaving at 121ºC for 20 minutes.

The ferrous iron (5.6 g/L Fe^{2+}) was supplemented as Fe^{2+} stock solution containing 22.5 g/L Fe^{2+} to all media of batch bottle experiments (batch assays, activity batch assays and shake flask controls). During the addition of $FeSO_4 \cdot 7H_2O$ to the Milli-Q water, the pH of the solution was maintained between 1.5 and 1.7 and adjusted to 1.7 of the final stock solution to prevent chemical iron oxidation and iron precipitation. The ready solution was then sterile filtered with 0.2 µm polyethersulfone membrane (VWR, International, North America) and stored at 4 °C in dark.

The iron oxidizing cultures of the pressure reactor were supplied with ferrous sulfate containing either 5.6 g/L Fe^{2+} or 10.0 g/L Fe^{2+}. The ferrous sulfate was added to the media that was already containing MSM, TES and MQ-water. The pH of the media was kept below 1.7 during ferrous sulfate addition.

5.3.2 Pre-cultivation of inoculum

Prior to the pressure experiments, the inoculum was pre-cultivated in two shake flasks with 100 mL working volume. Each shake flask contained medium and 10% (v/v) inoculum from the FBR. Before and after the addition of the inoculum with serological pipette, the pH was adjusted to 1.3 with concentrated H_2SO_4. The inoculated cultures were placed to an incubator shaker (Classic Series, New Brunswick Scientific, USA) and each time incubated at 35°C and 150 rpm (Figure 8). To ensure that the inoculum is each time in the same growth phase and has the same initial activity for the pressure experiments, the inoculum production shake flasks were always cultivated for 6 days and then used to inoculate the pressure reactor culture. The transfer of the culture to fresh medium to produce more inoculum when necessary took place on the 7^{th} day.

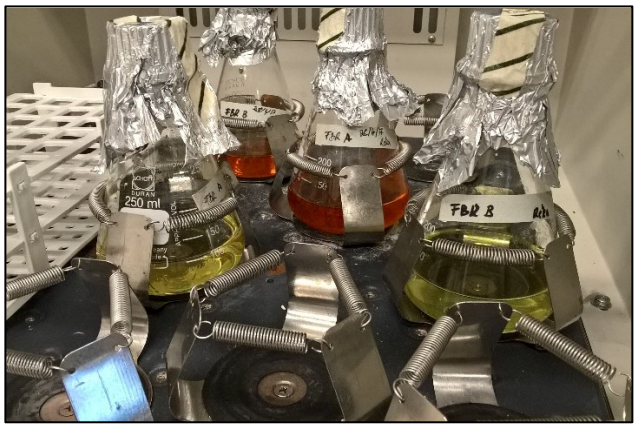


Figure 8: Shake flasks used to pre-cultivate iron oxidizing inoculum for the pressure experiments inside an incubator shaker operated at 35°C and 150 rpm.

5.3.3 Pressure experiments

Stirred tank pressure reactor (4524 bench top reactor, Parr Instrument Company, USA) was used for studying the effects of elevated pressures on biological and chemical iron oxidation. The Parr reactor (Figure 9) consisted of a titanium cylinder (total volume 2 L) that was placed to a heating blanket. The blanket was connected to a water supply that is automatically controlled. Beside the adjustment/maintenance of the temperature of the controlling unit, it is also used to set up the agitation speed inside the cylinder. The system was gastight, and the pressure inside the vessel was induced manually by a gas bottle (Aga, Finland) connected to the reactor's gas inlet by opening the gas supply valves. For the decrease of pressure, the gas outlet valve was used. The pressurized liquid samples were taken via the sampling port that was partly immersed to the culture broth. After the sampling, the pressue was adjusted back to the target level.

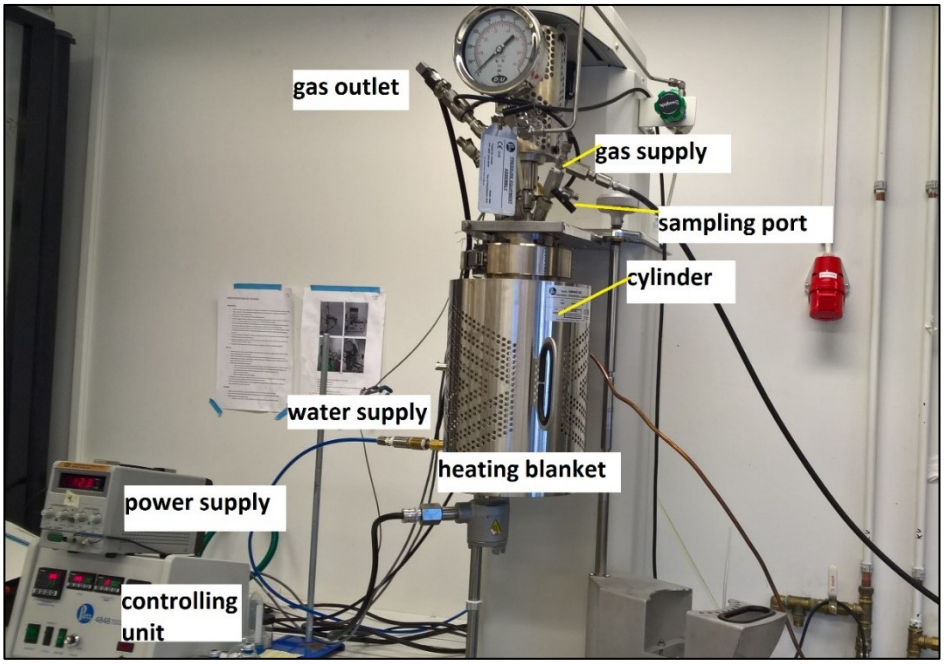


Figure 9: Parr Pressure Reactor used in the pressure experiments. The main components of the system are indicated in the figure. The 2 L cylinder with the culture (1 L) in it is inside the heating blanket. The power supply and controlling unit on the left were used for the control of temperature and agitation; and for the monitoring of the pressure inside the reactor. The pressure increase inside the cylinder was done via the gas supply and the decrease via the gas outlet valves. The sampling port also shown at the top was used for sample taking. The water used for the heating blanket was injected via the water supply pipe.

The cultures (1 L) used during the pressure experiments also contained 10% MSM, 1% TES, 10% (v/v) inoculum from inoculum production shake flasks, Milli-Q water and had pH 1.3. After adding all the required components to the cylinder of the pressure reactor (PR) and setting up the reactor, the pressure was increased to the desired level at a rate of 1 bar/min, and then the incubation parameters (summarized in Table 3) were either automatically (temperature and rpm) or manually (pressure) controlled. The duration of pressure experiments were 7 or 8 days. All chosen pressure levels (see Table 3) were used with and without microorganisms. The abiotic experiments were run for the detection of chemical oxidation of iron. After each experiment with the pressure reactor, the pressure was decreased using a rate of 1 bar/min (0.2 bar/12 sec), and from the biotic runs aliquots of culture (3x 10 mL) solution was used as inoculum for activity testing shake flasks (see section 6.3.4 for details). Liquid samples (10 mL per sampling) were taken from the pressure reacor every working day throughout the 7-8 days experimental runs (see Table 4 for sampling schedule).

Table 3: Different conditions used during the pressure experiments.

Experiment name	Inoculum	Fe^{2+} conc.	Pressure (bar) above atm	Temperature (°C)	Stirring (rpm)	Gas used for pressure increase
1 bar + (10 g/L Fe^{2+})	Yes	10.0	+1	35	150	Synthetic air
1 bar +	Yes	5.6	+1	35	150	Synthetic air
1 bar -	No	5.6	+1	35	150	Synthetic air
2 bar +	Yes	5.6	+2	35	150	Synthetic air
2 bar -	No	5.6	+2	35	150	Synthetic air
3 bar +	Yes	5.6	+3	35	150	Synthetic air
3 bar -	No	5.6	+3	35	150	Synthetic air
7 bar +	Yes	5.6	+7	35	150	Synthetic air
7 bar -	No	5.6	+7	35	150	Synthetic air
15 bar +	Yes	5.6	+15	35	150	Synthetic air
15 bar -	No	5.6	+15	35	150	Synthetic air
30 bar +	Yes	5.6	+30	35	150	Synthetic air
30 bar -	No	5.6	+30	35	150	Synthetic air
40 bar +	Yes	5.6	+40	35	150	Nitrogen
40 bar -	No	5.6	+40	35	150	Nitrogen

* The "+ " sign after the bar indicates the culture with inoculum and the "-" sign without.

Table 4: Sampling schedule for the different analyses and activity tests done throughout the pressure experiments.

Tests performed	Before pressure increase	After pressure increase	During fixed pressure (7-8 days)	Before pressure decrease	After pressure decrease
pH	yes	yes	every weekday	yes	yes
DO	yes	yes	every weekday	yes	yes
ORP	yes	yes	every weekday	yes	yes
Fe^{2+}	yes	yes	every weekday	yes	yes
Fe_{tot}	yes	yes	3 times/week	yes	yes

In most of the experiments 5.6 g/L Fe^{2+} was used, although one experiment (experiement 1 in Table 3) was conducted at initial Fe^{2+} concentration of 10 g/L. The pressure increase of the 40 bar experiment was done by using nitrogen. This case the 1% O_2 and 0.01% CO_2 and 99% N_2 were initially added to the culture. The addition of O_2 and CO_2 was done by gas syringe via the exhaust gas port prior the pressure increase. The O_2 volume was chosen to have maximum of 10 mg/L DO in the culture. The increase of pressure was done by nitrogen gas and no O_2 or CO_2 were added during the experiement.

5.3.4 Activity testing

After each inoculated pressure experiment (1 bar+ test, 1 bar+, 2 bar, 3 bar, 7 bar+, 15 bar+, 30 bar+ and 40 bar+), activity test in batch bottles (three replicates) containing media with 10% MSM and 1% TES as well as 10% (v/v) PR culture as inoculum was carried out at atmospheric pressure. To distinguish between the biotic and abiotic iron oxidation in the activity testing shake flasks, one negative control with no inoculum from the PR was also prepared each time. The working volume of the activity testing shake flasks were 100 mL and the initial pH of the cultures 1.3. To have comparable results between the pressure exposed (PR inoculum) and non-exposed cultures, activity testing shake flasks (3 inoculated + 1 negative control) were twice inoculated from the 6 days old inoculum of the inoculum production shake flasks. Each activity testing shake flasks were incubated in an incubator shaker (Classic Series, New Brunswick Scientific, USA) at 35°C and 150 rpm for 7-8 days and the daily (total 5 times) sampling volume was 3 mL.

5.4 Growth of biomass for pilot operation

5.4.1 Medium (FIGB feed solution)

Two different iron(II) concentrations were used during the different biomass production methods. The two concentrations used were 10 g/L or 5 g/L. The solution with 10 g/L Fe^{2+} was supplied to the FBR and added initially to the batch assays with activated carbon (AC), glass stirred tank reactor, bucket-type stirred tank reactor and semi-pilot reactor. The media transfers were done with 5 g/L Fe^{2+} in case of all biomass production methods.

Growth medium used for the biomass production consisted of 0.35 g/L $(NH_4)_2HPO_4$, 0.05 g/L K_2CO_3, and 0.05 g/L $MgSO_4$ in tap water. Before the addition of the nutrients, the pH was adjusted to ~1.3 by adding 3.5 mL/L (with 10 g/L Fe^{2+}) or 3.0 mL/L (with 5 g/L Fe^{2+}) of concentrated H_2SO_4. Once the nutrient solution was ready, 49.8 g/L (to reach 10 g/L Fe^{2+}) or 24.9 g/L (5 g/L Fe^{2+}) of $FeSO_4 \cdot 7H_2O$ was added and the solution mixed until all the $FeSO_4 \cdot 7H_2O$ was dissolved.

5.4.2 Means of biomass cultivation

The Figure 10 visualizes the the different means of biomass production used and the biomass flows between them. The inoculum for the small-scale AC bounded biomass production (batch assays, bucket-type stirred tank reactor, glass stirred tank reactor) was taken from the FBR´s effluent. All the biomass generated by these small-scale means was used as inoculum of the semi-pilot reactor. The upscaled biomass from the semi-pilot was completely transferred to the pilot reactor.

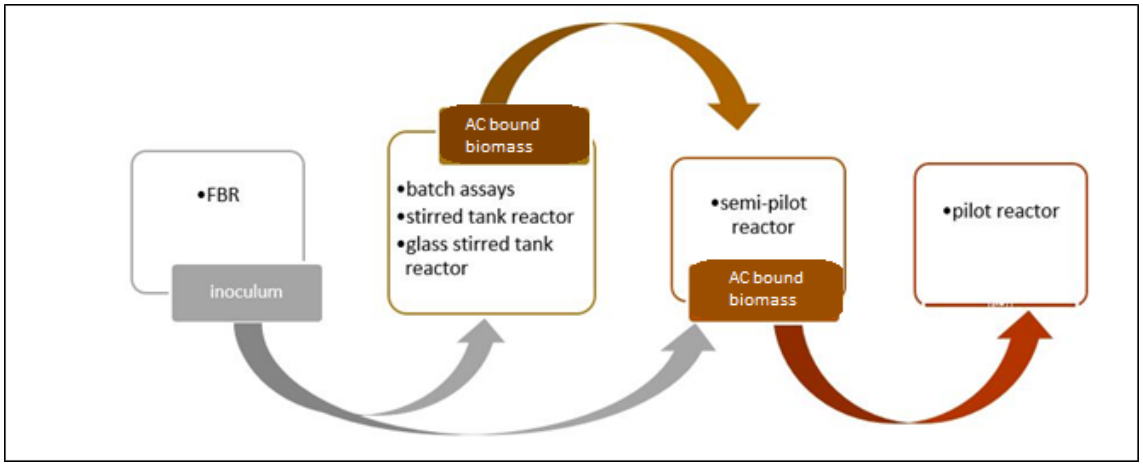


Figure 10: Process diagram of the AC bounded biomass production from laboratory to pilot-scale

Fluidized bed reactor (FBR)

The biological reactor system used for the incubation of enrichment culture and also partly biomass production consisted of an FBR, a heating blanket, an aeration unit, a recirculation pump and feed pump which were connected by rubber tubing (Figure 11). The FBR and the aeration unit had working volumes of approximately 900 mL. Granular activated carbon (FILTRASORB 200) was used as carrier material in the FBR. The fluidized bed volume and carrier fluidization were adjusted to 400 mL and ~10%, respectively. The AC was maintained in the FBR by using a larger and many small glass beads at the lower part of the reactor.

The operation of the FBR was conducted in the following manner: The iron (II) solution (feed) was supplied to the FBR via a feeding pump (50/60 Hz, Masterflex® Cole Palmer Instrument Company) to the recirculation pipe with iron (III) solution in it. This iron mix then entered the FBR and the remaining iron(II) was oxidized within the activated carbon layer and the iron(III)-rich solution left the FBR via its outlet at its top. Some part of this iron(III)-rich solution was recirculated via pump (50/60 Hz, Masterflex® Cole Palmer Instrument Company) back to the FBR and some left the system via an outlet at the top of the aeration unit (recirculation reactor in the Figure 12).

The recirculation pump flow was adjusted to 4.5 L/min and the feeding to 0.4-1.2 L/min depending on the need for the effluent for the biomass production. The temperature of the FBR was maintained at 35±2℃ and the DO above 3.0 mg/L. The pH of the culture was maintained between 1.4 and 1.7 by adjusted the pH of the feed solution (medium). The hydraulic retention time (HRT) during the inoculant and biomass production was 9.6-12.5 and 4.3-4.2 respectively. The DO, pH and ORP of the FBR were measured weekly.

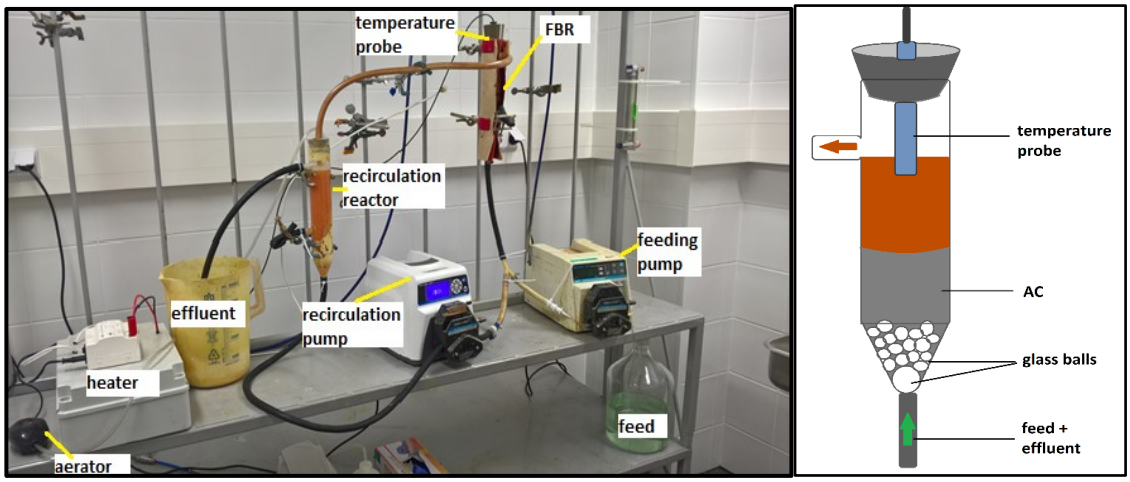


Figure 11: FBR system (left) and schematic figure of the FBR unit (right). The FBR system consisted of the FBR unit, aeration unit, feed tank, effluent tank, feeding pump and recirculation pump. The mixed acidophilic culture was bounded to activated carbon that was kept inside the FBR with the help of glass beads and the temperature was controlled with a heating blanket and temperature probe immersed in the culture solution.

Batch assays with AC

These assays were used during the small-scale AC bound biomass production. Altogether five 1000 mL (working volume 400 mL) and twelve 250 mL (working volume 100 mL) bottles were used. The preparation of the assays was conducted as follows. The AC was let to soak in tap water overnight and the next day, 50% (v/v) AC was placed to the bottles. After the AC addition, 40% FIGB feed solution (10 g/L Fe^{2+}) and 10% (v/v) inoculum (effluent of the FBR) were added by serological pipettes. The bottles were inocubated in an incubator shaker (Innova 44, New Brunswick Scientific, USA) at 35°C and 150 rpm (Figure 12). Once a week most of the liquid media was replaced with fresh medium having similar composition as the FIGB feed solution (5g/L Fe^{2+}) and the cultivation was run for 1 month. The medium-replacements were performed when the assays had dark orange color, so the iron(II) oxidation efficiency was above ~80%.

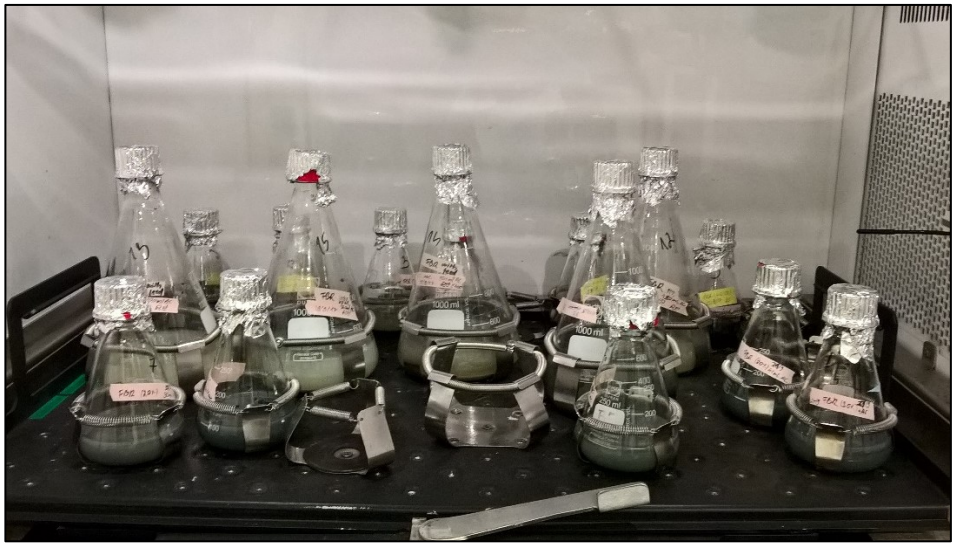


Figure 12: AC bound biomass production in batch bottles with a volume of 250 mL and 1000 mL. The temperature and mixing rate used were 35°C and 150 rpm, respectively. This photo was taken right after the media change. The grey color was due to the AC.

Bucket-type stirred tank reactor

Another mean to generate AC bound biomass was using bucket-type stirred tank reactors (Figure 13). Altogether three reactors were used. Two of them had a maximum volume of 45 L (approx. 20 L working volume) and one 30 L (approx. 14 L working volume). As shown in Figure 14, each reactor consisted of a polyethylene bucket; a polytetrafluoroethylene agitator which was stirred with a motor; two glass aerators attached to the opposite sides of the bucket´s inner wall and a polyethylene cover at the top. Each reactor was supplied with air by using two (100 and 75 L) aquarium aerators (Marina, Hagen, China).

Figure 13: Bucket-type stirred tank reactors used for AC bounded biomass production. The AC with the FIGB (10 g/L) solution and inoculum was stirred at 50 rpm and aerated via two glass aerators (175 L/min).

Prior to the start of the biomass production, the AC was soaking in tap water for overnight. The wet 30% (v/v) AC (FILTRASORB 200) was put to each of the buckets with the agitator and aerators. After the AC addition, 10% (v/v) effluent from FBR as inoculum and 60% FIGB feed solution (10 g/L Fe^{2+}) was also added to each bucket. The stirring of the culture was adjusted to 50 rpm and the aeration to ~175 L/min. The cultivation temperature was 25±2°C. The 50% (v/v) liquid media was transferred with FIGB solution (5 g/L Fe^{2+}) once a week in the first two and twice a week during the last week of the 3-week cultivation. After the cultivation, the solid biofilm-coated AC was collected to plastic buckets with a lid.

Glass stirred tank reactor

Glass stirred tank reactor was used the last mean of small-scale AC bounded biomass production. The biomass generated by the glass stirred reactor was used as part of the inoculum of the semi-pilot reactor. The Figure 14 shows the glass stirred tank reactor system. The system consisted of a mixer with stainless steel agitator, a 2 L glass tank, aeration pipe and a heating unit. The tank had double wall and warm water was circulated inside them. Initially 50% (v/v) overnight soaked in water AC, 10% (v/v) inoculum (effluent of FBR) and 40% FIGB feed solution (10 g/L Fe^{2+}) were added to the glass tank

with the agitator. The working volume of the reactor was 1.4 L, the agitation 60 rpm and the temperature inside 35°C. The amount of aeration was adjusted in a way to achieve visually enough bubbling in the liquid phase. Some part of the media evaporated during the operation, so before each media transfer, the evaporated liquid volume was replaced with tap water. Throughout the 2.5-week operation, the media was transferred every 4-5 days once it had turned to strong dark orange color. During each transfer, the evaporated liquid was refilled with tap water and then 50% of the liquid media has been replaced with FIGB feed solution with 5 g/L Fe^{2+} concentration. The concentration was reduced from 10 g/L for the prevention of toxic levels of Fe^{3+}. The initial culture before the addition to the reactor had pH 1.3. After the 2.5 weeks operation, the liquid media removed and the AC collected to a plastic bucket with a lid.

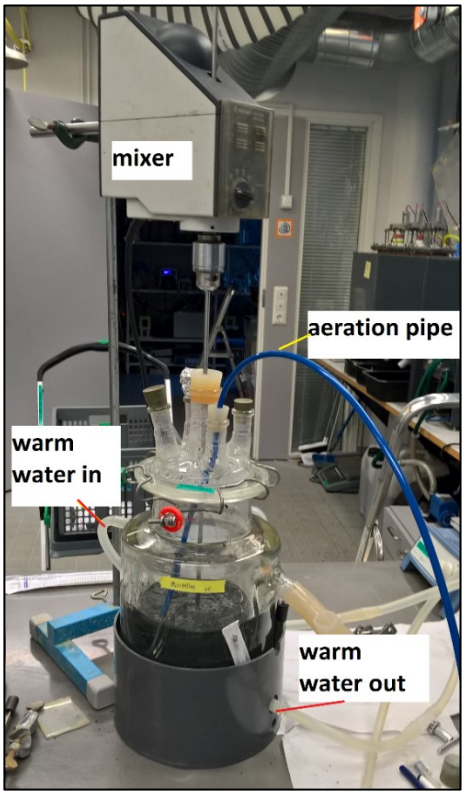


Figure 14: Glass stirred tank reactor used for biomass generation. The reactor was aerated via a plastic pipe, agitated at 50 rpm and its temperature maintained at 35°C by circulating water around the reactor.

Semi-pilot reactor

The semi-pilot reactor was used as the last mean of AC bound biomass production for the pilot reactor. This reactor (Figure 15) consisted of a 1 m^3 polyethylene tank with open top, a stirrer, air supply (HVAC, ABB, Sweden) via aeration ring and a valve at the bottom of the reactor. The working volume of the reactor was approximately 600 L. The starting culture contained 35% (v/v) AC and 65% (v/v) liquid media (1:2 ratio). This part of the biomass production took place at the Geological Survey of Finland (GTK Mintec), Outokumpu premises.

As initial step of the semi-pilot operation, 320 L medium having the same composition as the FIGB feed solution (10 g/L Fe^{2+}) was added to the reactor, aeration set to ~60 Hz, slow stirring started and 75 kg of AC (FILTRASORB 200) was slowly poured to the medium. During and after the AC addition, the stirring speed was adjusted to properly mix the solid matter. Once the bubbling caused by AC addition had stopped, 80 L of FBR effluent and 14 L AC bounded biomass (produced by the various small-scale means) were added as inoculum to the reactor. Once the culture was ready, the pH was adjusted to 1.5 with pH 1.0 tap water. After half day operation, additional 25 kg of AC was added to the reactor and the pH adjusted back to 1.5 with acidic tap water (pH 1.0). The final AC: media ratio was 3:5 (v/v).

The ferrous iron concentration of the culture was measured daily and once the iron oxidation efficiency had reached 80-90%, the evaporated liquid volume was filled back with tap water. Then half of the medium was replaced with fresh FIGB feed solution (with Fe^{2+} concentration either 5 or 10 g/L, depending on the Fe(tot) concentration inside the reactor). Besides the ferrous iron concentration, the pH, DO, ORP and ferric iron concentration of the culture were also monitored daily. The biomass production was run for 1 month in the semi-pilot reactor. At the end of the biomass production, the liquid media was completely replaced with tap water (pH 2.0) to remove most of the Fe(tot). This washing step was performed three times. After the removal of the last washing solution, the AC bounded biomass was manually collected to 20 L polyethylene canisters (14 L to each), supplied with 6 L FIGB solution (with 2 g/L Fe^{2+}) and stored at 7-30°C. In total 196 L AC bounded biomass was produced.

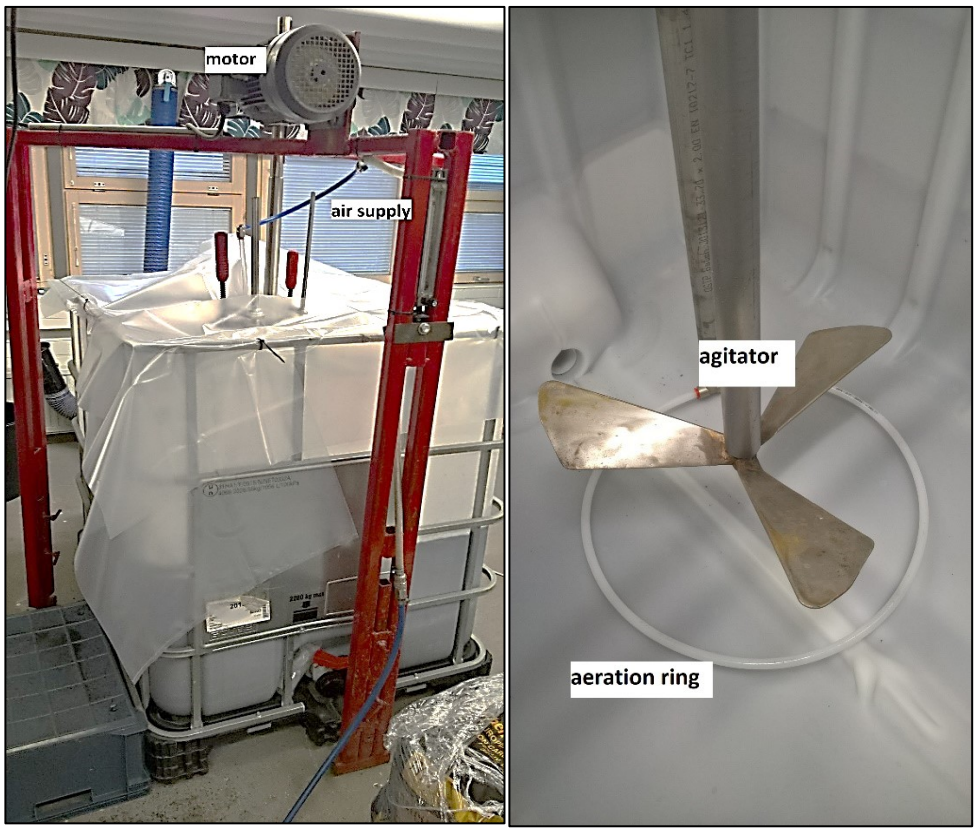


Figure 15. Semi-pilot reactor from outside (on left) and from inside (on right). The reactor was used for larger-scale AC bounded biomass production to obtain enough biomass for the pilot reactor.

Pilot reactor

The purpose of the pilot reactor is demonstrate applicability of *in situ* bioleaching in mine enviroment. The pilot reactor (Figure 16) is a part of a bioleaching system and it has been designed by Hatch Ltd. The reactor´s total volume is 455 L and its working volume is ~450 L to prevent overflow of the liquid medium. Once the bioleaching-phase will run, the bioreactor will be used for the regeneration of ferric iron. The ferrous iron separated from the PLS will be recirculated to the bioreactor from its bottom. The Fe^{2+} will be oxidized within the AC bounded biomass and the Fe^{3+}-rich solution will leave the reactor by overflowing form the top. This overflowing solution will be reused in the bioleaching process.

Until starting the *in situ* bioleaching, the reactor is operated in semi-batch mode to maintain activity of the bacterial biofilm. The starting of the pilot reactor was done as follows: All the AC bounded biomass (196 L) produced in the semi-pilot reactor was added to the reactor together with its liquid medium (84 L) containing ferric iron solution

(~2 g/L Fe^{3+}). At the same time, 56 L fresh wet AC, 24 L FIGB solution (with 2 g/L Fe^{2+}) and some water (pH 2.0) to reach the 450 L working volume were added. As last step, 500 g $FeSO_4 \cdot 7H_2O$, 225 g $(NH_4)_2SO_4$ and 25 g KH_2PO_4 were also added to the pilot reactor. Once the culture was ready, the aeration (100 L/min) was turned on and the pH of the culture was adjusted to 1.5 with concentrated H_2SO_4. Once a week 50 L of the liquid medium is drained away and replaced with 100 L tap water via the bottom of the reactor. The 50 L volume difference is due to evaporation. After adding water, 500 g $FeSO_4 \cdot 7H_2O$ (~ 1 g/L Fe^{2+}) and 1 L of ammonium sulfate and potassium phosphate solution (225 g/L $(NH_4)_2SO_4$ and25 g/L KH_2PO_4) are added from the top of the reactor. The pH of the reactor is continuously monitored and maintained at 1.6 by adding H_2SO_4 when it is needed. The temperature of the reactor is not controlled. The temperature of the reactor is kept at 29±2°C, which is the ambient temperature in the underground chamber at the Rudna mine (KGHM Polska Miedz), Poland where the reactor is located.

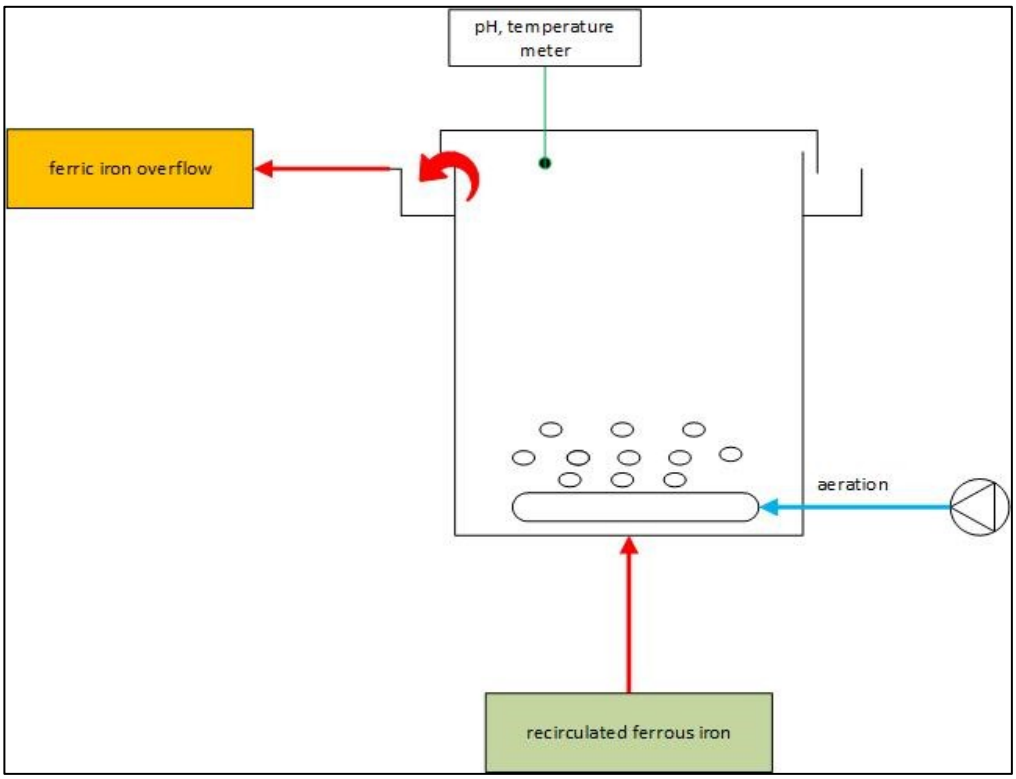


Figure 16: BIOMOre pilot reactor (modified from Zeton B.V. Process and Instrumentation diagram, Project 1601). The bioreactor tank is filled with AC bounded biomass in solution with ferrous- and ferric iron. The recirculated ferrous iron comes from the PLS after metal removal. The ferrous iron solution enters the bioreactor from the bottom where it is oxidized, and then leaves as ferric iron through the top of the reactor. The mixing of the biomass is done by using high aeration from the bottom. Theodore Ineich from Hatch Ltd. gave permission to modify and use the process drawing of the bioreactor

5.5 Analytical methods and calculations

5.5.1 Measurement of DO, redox and pH

DO, redox and pH were measured from non-filtered samples. During the pressure experiments and small-scale biomass production, the DO of the samples was measured with HQ40d multi meter (Hach, USA) right after sampling to avoid loss of oxygen from the samples. The major loss of oxygen probably occurred of the samples from the pressure reactor because their measurements in unpressurized environment. The determination of redox potential was done by pH 315i meter (WTW, Germany) with BlueLine 31 Rx (Ag-Ag) redox electrode. For the detection of pH, pH 3210 meter (WTW, Germany) with SenTix41 pH-electrode (WTW, Germany) was used. The pH electrode was calibrated before the measurements by using pH buffer standards with pH 4 and 2.

The DO of the semi-pilot reactor was measured straight from the reactor and the pH and redox right after sampling. The equipment used were Sension+ DO6, Hach DO meter; Metrohm 826 pH mobile pH meter and Metrohm 744 pH meter (comb. Pt-ring electrode) for the measurement of ORP. The Fe^{2+} and Fe_{tot} were measured with UV/VIS Lambda spectrometer (PerkinElmer).

The pH measurement of the pilot operation is done automatically. A Jumo Techline with a Jumo Aquis 500 pH meter was installed to the bioreactor by Hatch Ltd. The meter records the actual pH every 5 minutes. The electrode is daily calibrated with buffer solutions with pH of 4 and 1. The redox potential is measured by Ag/AgCl electrode.

5.5.2 Ferrous iron concentration

For the measurement of the ferrous iron concentration, the 3500-Fe ortho-phenantroline method (American Health Association, APHA, 1992) was used. Prior to the analysis, the samples were filtered with 0.45 µm Chromafil Xtra PET -45/25 polyester filters (Macherey-Nagel GmbH and Co. KG, Germany) and diluted 1000-times with 0.07 M HNO_3. From the diluted sample 1 mL was mixed with 2 mL phenantroline solution, 1 mL ammonium acetate buffer, 0.9 mL Milli-Q water and 0.1 mL 37% HCl. The absorbance of the samples was measure at 510 nm using Ordior UV-1700 Pharma spectrophotometer (Shimadzu, Japan), using 0.07 M HNO_3 as the zero sample. The samples were put right after their preparation into quartz-cuvette and their absorbance values measured within 5 minutes after mixing all the reagents together. After the measurements, the absorbance values were converted to concentrations by using the standard curve.

For the preparation of standard curve, 200 mg/L Fe^{2+}-stock solution was made by dissolving 1.404 g $Fe(NH_4)_2(SO_4)_2*6H_2O$ in 20 ml concentrated H_2SO_4 and 50 mL Milli-Q water. The solution was then titrated until a faint pink color with 0.1 M $KMnO_4$ and finally diluted to 1000 mL. Then the stock solution was further diluted to have 5 different concentrations within the range of 0.05-20 mg Fe^{2+}/L. The standard curve from the 5 Fe^{2+} concentrations was prepared prior the ferrous iron measurement from the sample.

The Fe^{2+} concentrations of the samples taken from the semi-pilot were measured with Lambda 35 UV/VIS (PerkinElmer) right after sampling. This study does not contain any Fe^{2+} concentration data of the pilot reactor.

5.5.3 Total iron concentration

All the samples from the pressure reactor and batch bottles (stored at 4-8°C) were diluted 4000-times with 0.07 M HNO_3 and their total iron (Fe(tot)) concentrations measured by using Atomic Absorption Spectrometry (Perklin Elmer Precisely, Analyst 400 Atomic Absorption Spectrometer).

The Fe(tot) of the samples taken from the semi-pilot were measured with Lambda 35 UV/VIS (PerkinElmer) right after sampling. This study does not contain any Fe(tot) concentration data of the pilot reactor.

5.5.4 Iron oxidation

The efficiency of iron oxidation process was calculated by using the Equation 12 (Nurmi et al., 2009a). In the Equation 12 the Fe_{in}^{2+} is the initial and the Fe_{out}^{2+} is the actual ferrous iron concentration.

(12)

$$iron\ oxidation\ efficiency = \frac{Fe_{in}^{2+} - Fe_{out}^{2+}}{Fe_{in}^{2+}} \cdot 100\%$$

The rate of iron oxidation based on the ferrous iron concentrations was calculated by using the Equation 13. In the Equation 13 the *initial Fe^{2+} conc.* is the concentration measured on the day 0 and the final *Fe^{2+} conc.* is concentration on the last day of the period taken into account.

(13)

$$Fe^{2+}\ oxidation\ rate = \frac{initial\ Fe^{2+}\ conc. - final\ Fe^{2+}\ conc.}{time\ (duation)}$$

6. RESULTS

6.1 Effect of pressure

6.1.1 Effect of iron concentration on iron oxidation at 1 bar above atmospheric pressure

In the first part of the pressure experiments, the effect ferrous iron concentration on iron oxidation was tested at 1 bar overpressure. The tested Fe^{2+} concentrations were 10 g/L and 5.6 g/L. Considering the stoichiometry of iron oxidation, 0.8 g/L (800 mg/L) and 1.4 g/L (1433 mg/L) oxygen is needed to fully oxidize 5.6 g/L and 10 g/L Fe^{2+}, respectively.

In the experiments the Fe^{2+} oxidation was only partial at initial Fe^{2+} concentration of 10 g/L (1 bar+ test experiment) whereas most of it was oxidized at initial concentration of 5.6 g/L (1 bar+ experiment) (Figure 17 a). After 9 days, the oxidation efficiency was 44% in the 10 g/L and 84% in the 5.6 g/L experiment. The iron oxidation rate was similar i.e., 0.52 and 0.53 g/L/d of the 10 g/L and 5.6 g/L experiments, respectively. Fe^{2+} was steadily or with minor variations decreasing during the 10 g/L and 5.6 g/L experiments. The Fe_{tot} (Figure 17b) with both experiments remained fairly constant throughout the 9-10 days runs. The Figure 17c shows that the DO concentration decreased in the 5.6 g/L experiment from 9 to 3 mg/L during the first two days of experiment. From the 2nd day to the 9th day, the DO concentrations of both runs changed similarly and had only small variations. The pH in both experiments (Figure 17d) developed identically. It increased with both experiments approximately 0.25 in 7 days.

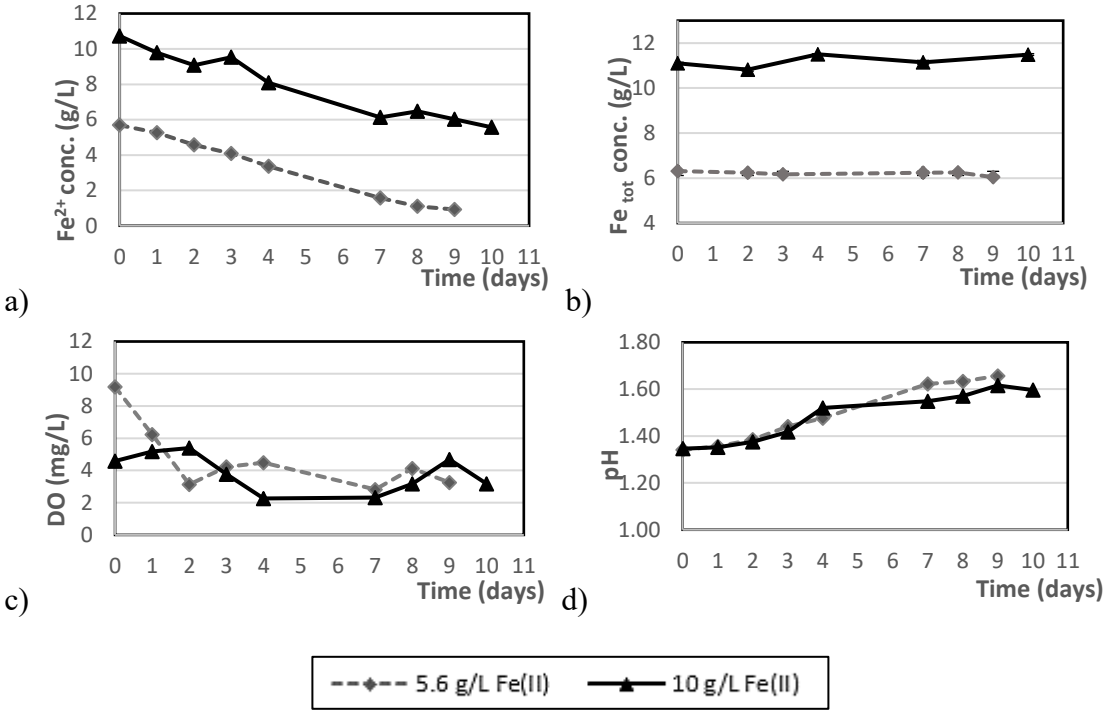


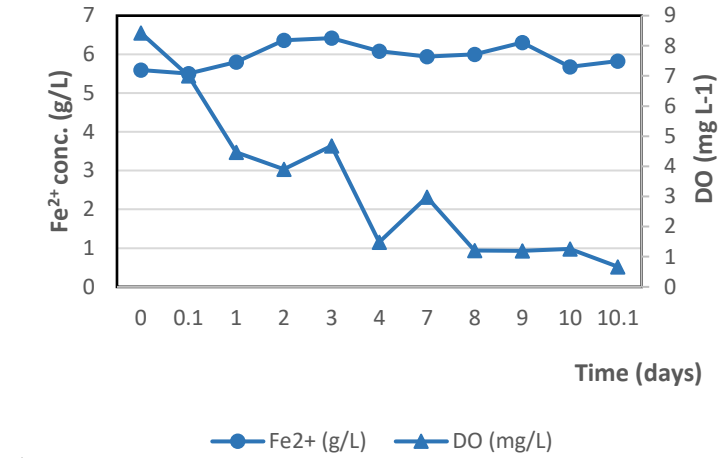
Figure 17: Effect of iron concentration on iron oxidation rate and efficiency at 1 bar overpressure. The a) shows the concentration of ferrous iron, b) concentration of total iron, c) concentration of dissolved oxygen and d) development of redox potential by time during the experimental runs. Both pressure experiments were run in 35°C±1°C and 150±2 rpm for 9-10 days.

The results show that the initial 10 g/L Fe^{2+} concentration was too high for complete iron oxidation by the iron oxidizers within 10 days. Therefore, 5.6 g/L Fe^{2+} was used in the following experiments.

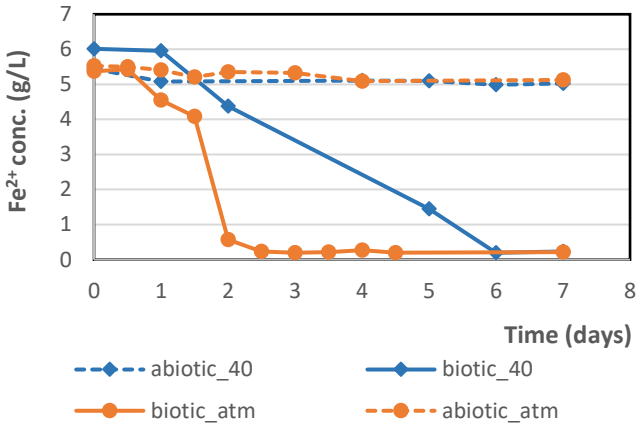
6.1.2 Effect of high pressure and the rate of pressure increase/decrease at low oxygen partial pressure

At this part of the study, the effect of the rate of pressure increase and decrease on the iron oxidizers was tested. The pressure used was 40 bar above atmospheric pressure and the rate of increase/decrease was 10 bar/min.

At +40 bar pressure no biotic or abiotic iron oxidation occurred (Figure 18a). The Fe^{2+} concentration remained between 6.3 and 5.5 throughout the 10-day experiment. Also the ferrous iron concentration slightly increased (+0.7 g/L) from its initial concentration. The abiotic iron oxidation was halted because of too low DO concentration. The DO was only ~1.5 mg/L on the 4^{th} day of experiment. Although no biotic iron oxidation took place at +40 bar, the activity testing shake flaks with inoculum from the +40 bar experiment (Figure 19b) showed survival of the acidophiles during the +40 bar experiment. After the pressure experiment, the iron oxidizers (see biotic_40 in Figure 18b) oxidized ferrous iron but on a slower rate than the control cultures inoculated with microorganisms from non-pressurized environment (see biotic_atm). The mean Fe^{2+} oxidation rate was 0.97 g/L/d in 6 days period of pressure exposed shake flasks and 1.73 g/L/d of biotic control cultures in 3 days.



a)



b)

Figure 18: On the top (a) is the ferrous iron oxidation and changes of dissolved oxygen (DO) during the biotic +40 bar pressure experiment. Down (b) is the biotic Fe^{2+} oxidation in the activity testing shake flasks (SF) after the 40 bar+ experiment (biotic_40) and in activity testing SF with inoculum originating from non-pressurized culture (biotic_atm). In (b) also the abiotic runs (abiotic_40 and abiotic_atm) are included. Oxygen was added only initially to the pressure runs while it was continuously coming from the air to the shake flasks at atmospheric pressure.

The results show that the high rate (10 bar/min) speed of pressure increase/decrease had minor effect on the iron oxidizers based on the difference between the biotic controls (biotic_atm) and +40 bar activity testing shake flasks (biotic_40) result. However, more moderate 1 bar/min rate of increase/decrease was used in the following experiments. Considering the fast consumption of initial O_2 and the impossibility of O_2 addition to the pressure reactor, air was used for the pressure increase in the later experiments.

6.1.3 Effect of elevated pressures in aerobic conditions

Different pressures (+1, +2, +3, +7, +15 and +30 bar) induced by air were applied on the iron oxidizing culture to see their effect on iron oxidation.

Although initially there was some minor biotic iron oxidation in all +7, +15 and +30 bar pressure experiments based on the differences between the biotic and abiotic experiments, the parallel development of the iron oxidation curves with and without inoculum shows the dominancy of abiotic iron oxidation at these pressure levels (Figure 19a). The redox potential also confirms similar Fe^{2+}/Fe^{2+} ratios (Figure 19c). At biotic and abiotic conditions, the ferrous iron oxidation rates (Table 5) at +15 bar (0.51 and 0.42 g/L/d) and +30 bar (0.58 and 0.52 g/L/d) were slightly different. The highest difference of iron oxidation rate within these three pressure levels was at +7 bar. The rate was 0.47 g/L/d and 0.28 g/L/d in the biotic and the abiotic experiment, respectively. These differences between the biotic and abiotic iron oxidation rates can be accounted for the initial biotic iron oxidation. The abiotic iron oxidation rate was highest at +30 bar. All elevated pressure levels enhanced the abiotic iron oxidation rates compared to the shake flask experiment at atmospheric pressure (Table 5). The abiotic iron oxidation rate increased by 9.3, 13.7 and 17.3 times by applying pressures +7, +15 and +30 bar, respectively. Considering the biotic iron oxidation, the oxidation rates were 3.6, 3.3 and 2.9 times reduced compared to the biotic control experiments at atmospheric pressure, by applying pressures +7, +15 and +30 bar, respectively. Although the abiotic oxidation is improved by increasing the pressure, but the rate of the increase slowed down after +15 bar. The DO concentration measured from the unpressurized samples was above or at least close to 10 mg/L throughout the pressure experiments at +7, +15 and +30 bar (Figure 20d). The activity testing shake flasks demonstrated that iron oxidation activity was inhibited at these three pressure levels induced by technical air, as iron oxidation was not detected in the shake flask cultures (Figure 20).

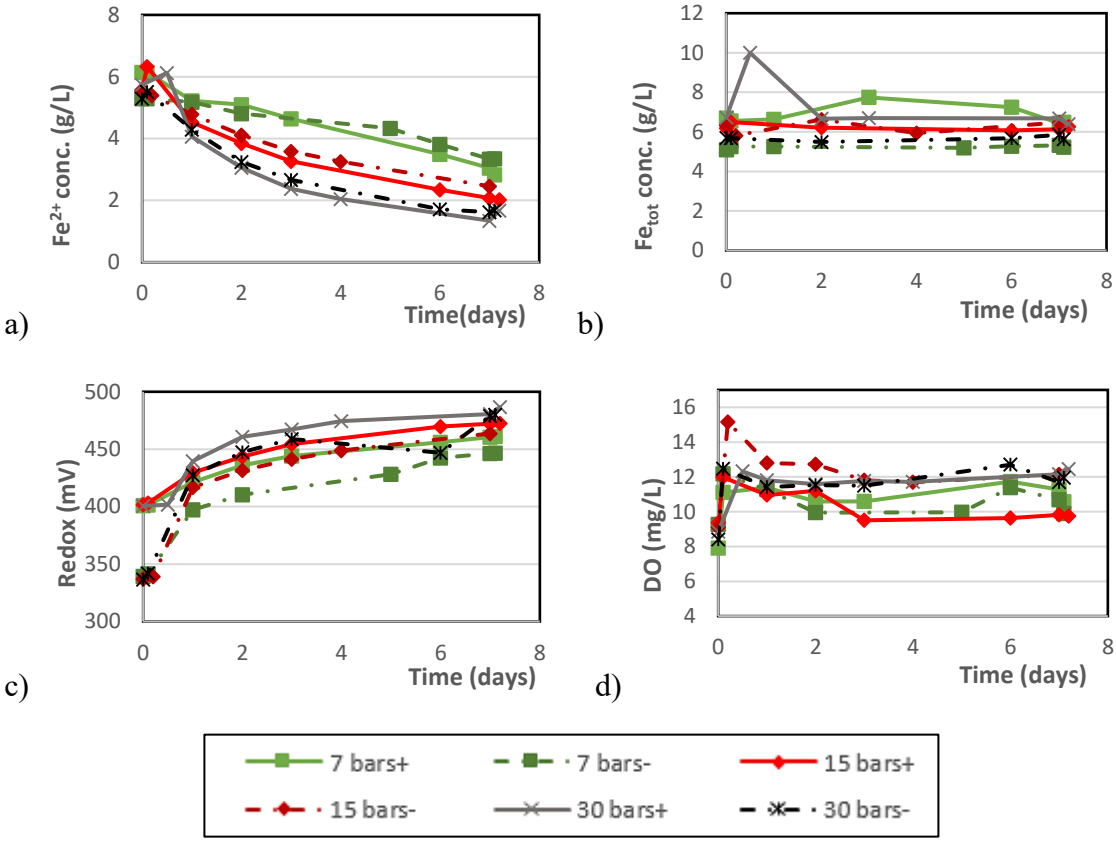


Figure 19. Biotic and abiotic pressure experiments at 7, 15 and 30 bar above atmospheric pressure. The Fe^{2+} oxidations (a), total iron concentrations (b), redox potentials (c) and DO concentrations (d) are shown. The + signs are indicating the biotic and the – signs the abiotic experiments.

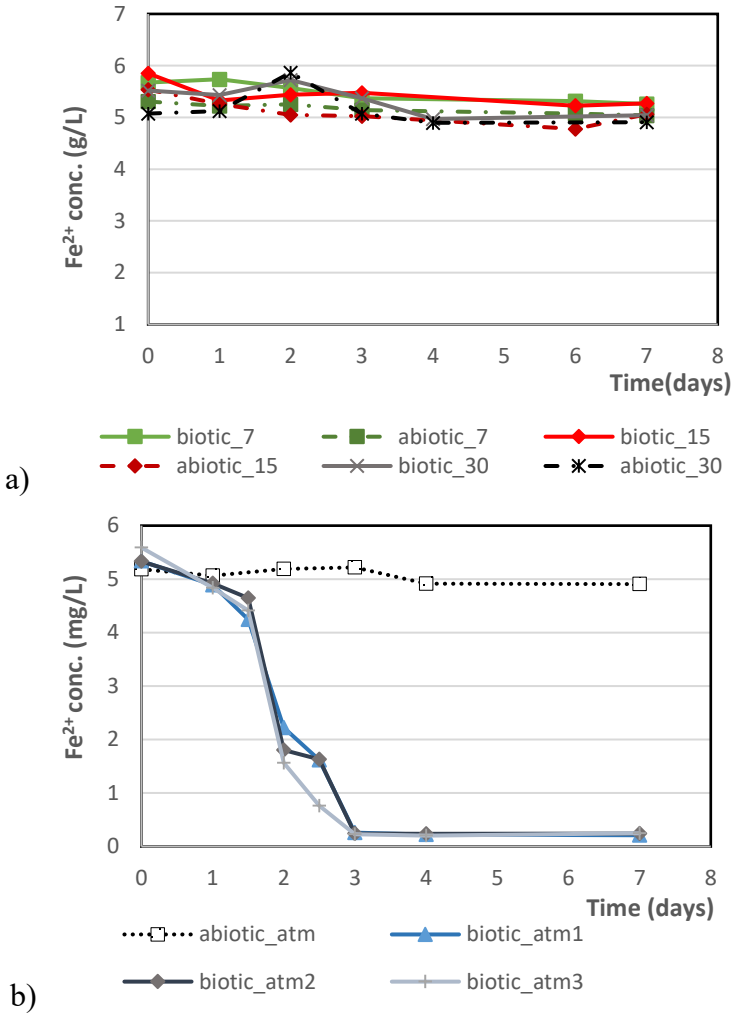


Figure 20: Iron oxidation in the activity testing shake flasks (a) with inoculum (10% v/v) from +7, +15 and +30 bar experiments (biotic_7, 15, 30) and without inoculum (abiotic_7, 15, 30) and (b) with 10% (v/v) inoculum from inoculum production shake flasks (biotic_1, 2, 3) and without inoculum (abiotic_atm).

Based on the results described above, the pressure levels ≥ +7 bar induced by technical air were inhibitory for the activity of iron oxidizing culture. To determine the pressure limit of the iron oxidizers, additional levels within +1 and +7 bar were tested. The selected levels were +2 and +3 bar which are similar that are typically used with traditional autoclave and pressure oxidation, respectively.

Table 5: Biotic and abiotic ferrous iron (Fe^{2+}) oxidation rates at the studied elevated pressure levels.

Total elevated pressure (bar)	Calculated O_2 partial pressure (bar) based on the elevated pressure	abiotic iron oxidation rate (g/L/d)	biotic iron oxidation rate (g/L/d)
atm*	-	0.03	1.7
+1	+0.21	0.20	0.59
+2	+0.41	0.22	0.73
+3	+0.63	0.19	0.78
+7	+1.47	0.28	0.47
+15	+3.15	0.41	0.51
+30	+6.3	0.52	0.58

* This refers to the control shake flask cultures at atmospheric pressure.

The experimental runs with +1, +2 and +3 bar showed biological iron oxidation, as demonstrated by the more significant decrease of Fe^{2+} concentration in the biotic compared to the abiotic runs (Figure 21a). While the abiotic runs at +1, +2 and +3 bar had iron oxidation efficiency of 23, 32 and 28% in 6 days, the biotic runs at the same pressure levels had oxidation efficiency of 72, 97 and 99%, respectively. The iron oxidation rates (Table 5) at these three pressure levels increased by the acidophiles. These improvements were +0.39 +0.51 and +0.60 g/L/d of the +1, +2 and +3 bar experiments, respectively. The total iron concentration of the different runs were slightly different (Figure 21b) and the +3 bar run had the highest total iron concentration. Compared to the biotic iron oxidation rate at atmospheric pressure obtained in 3 days, the oxidation rate was 2.8, 2.3 and 2.18 times lower of +1, +2 and +3 bar experiments, respectively. Considering the abiotic iron oxidation rate at atmospheric pressure, the rate increased by 6.7, 7.3 and 6.3 times by pressure levels +1, +2 and +3 bar, respectively. The steeper increase of pH and redox (Figure 21c and d) of the biotic runs compared to the abiotic ones also showed the microbial activity at all three pressure levels. The data (Figure 21 a, b, c and d) shows that the activity of iron oxidizing culture was similar at +2 and +3 bar and higher than at +1 bar. The DO concentrations measured from the samples that were taken out from the pressure reactor showed similar starting concentrations (9.4-8.5 mg/L) of all three biotic experiments. The DO was decreasing more during the +2 and +3 bar experiment than in the +1 bar which indicates higher iron oxidation activity in the those two higher pressure levels. In the abiotic experiments, the DO of the +1 bar run was fairly constant throughout the 7 days experiment while there were more variations in +2 and +3 bar experiments.

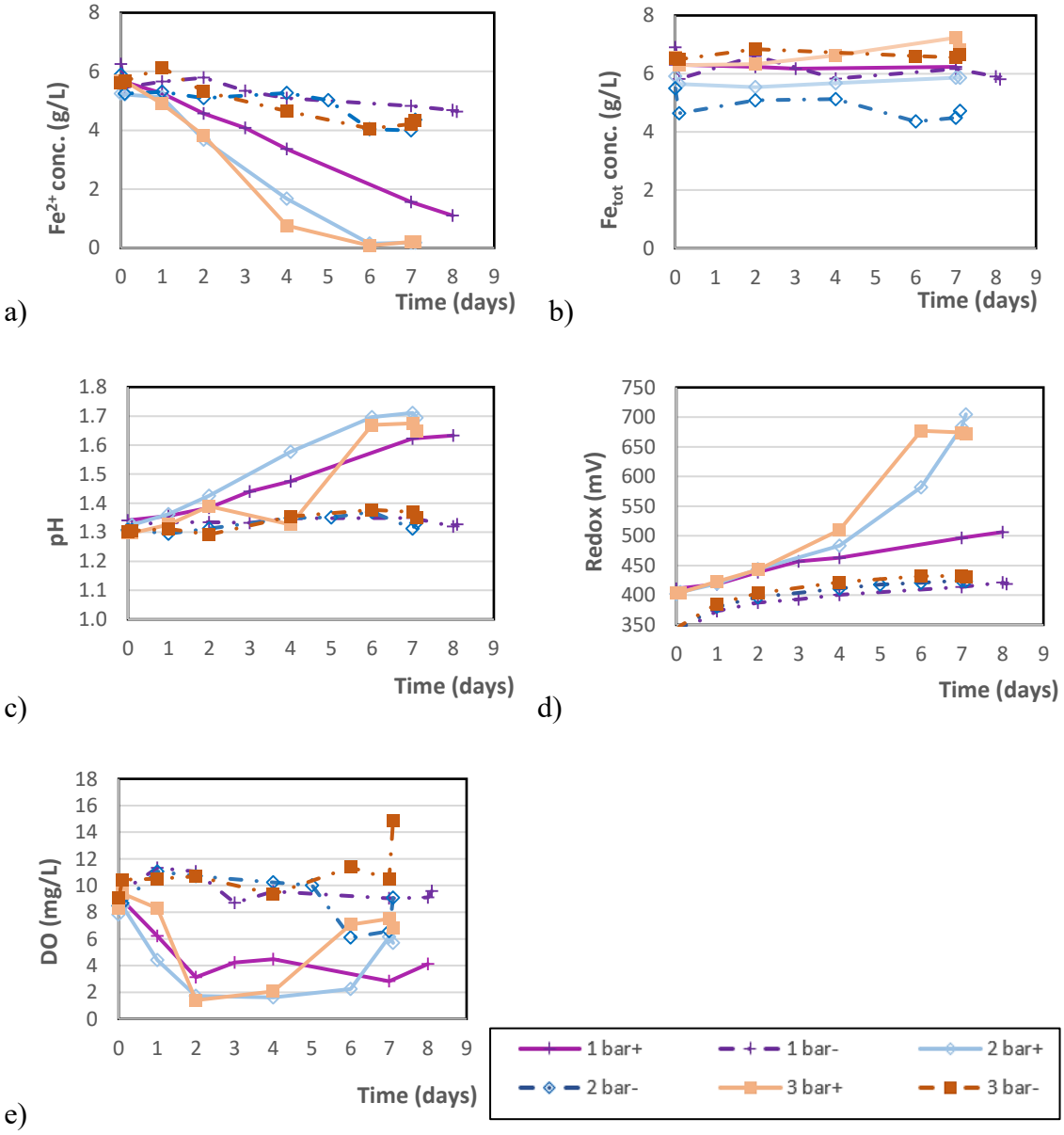


Figure 21: Biotic (indicated by plus sing) and abiotic (indicated by minus sign) pressure experiments at 1, 2 and 3 bar above atmospheric pressure. The Fe^{2+} oxidations (a), total iron concentrations (b), pH (c) redox potential (d) and DO (e) are shown.

The activity tests after the pressure experiments (Figure 22) did not indicate clear inhibition of the iron oxidation activity by the +2 and +3 bar pressure runs. although the iron oxidation rates of the +2 (biotic_2) and +3 bar (biotic_3) cultures were lower than that of the biotic control (biotic_atm) originating from unpressurized environment. The

iron oxidation efficiency reached 90% on the day 2, day 4 and day 4 in the biotic_atm, biotic_2 and biotic_3 shake flasks, respectively. The iron oxidation efficiency shows that the +2 bar was less harmful to the microbial activity than +3 bar. The activity testing was not run after the +1 bar experiment.

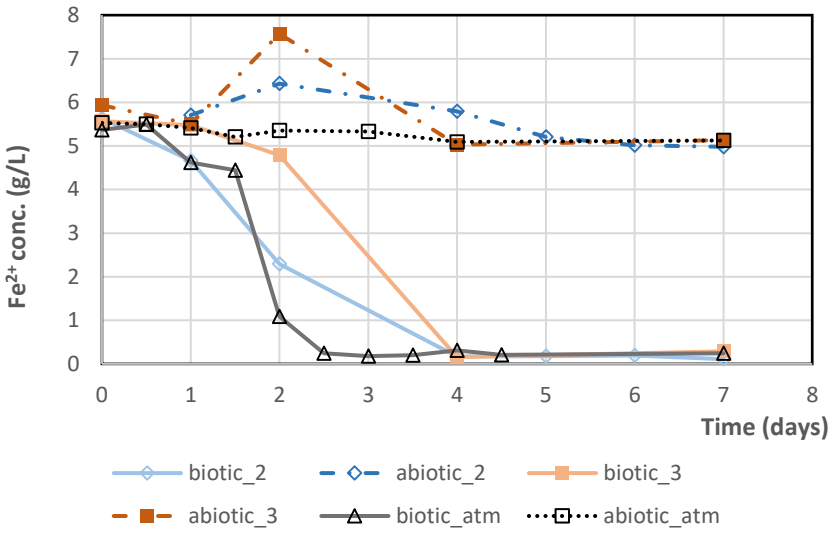


Figure 22: Fe^{2+} iron oxidation in the activity testing shake flasks (average from triplicate cultures) with inoculum (10% v/v) from the +2 (biotic_2), and +3 bar (biotic_3) experiments and with 10% (v/v) inoculum from inoculum production shake flask (biotic_atm). One abiotic control was also prepared at all tests.

The iron oxidation rates in the abiotic cultures (Figure 23a) increased with increasing pressure. The coefficient of determination (R^2) was > 0.94 which indicates strong correlation between the variables. The oxidation rates in the biotic cultures (Figure 23b) showed increase up to +3 bar. The biotic iron oxidation rates from +7 to +30 bar were similar to the abiotic controls indicating that mainly chemical oxidation took place at those levels of elevated pressure.

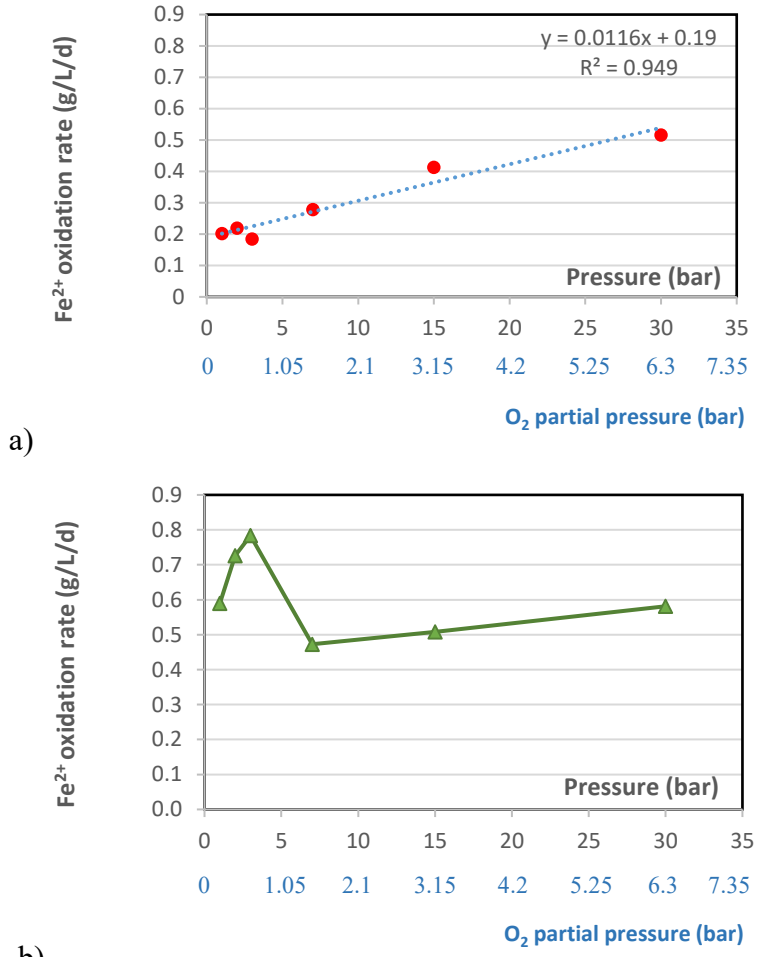


Figure 23: Fe^{2+} oxidation rates as a function of elevated pressure (a) in the abiotic and (b) biotic runs. On the horizontal axes the total pressures with respect to the atmospheric pressure are shown with black and the calculated O_2 partial pressure with blue text.

The results show, that the inhibitory pressure (or oxygen partial pressure) level considering the biotic iron oxidation is between +3 bar (0.63 bar O_2 partial pressure) and +7 bar (1.47 bar O_2 partial pressure). The results demonstrate that the aerobic abiotic iron oxidation at elevated pressures (e.g. existing at deep subsurface application) is possible if sufficient oxygen is provided.

6.2 Growth of iron oxidizing biomass at increasing scales

In the second part of this study, scaling up the AC bound biomass (culture with *L. ferriphilum and Sulfobacillus sp.*) growth from laboratory to pilot-scale operation was performed. The starting inoculum was effluent from the laboratory-scale FBR (containing ~5% iron oxidizers in planktonic state).

6.2.1 Laboratory-scale biomass production

Laboratory-scale AC bound biomass production aimed at production of enough biomass inoculum for a semi-pilot operation. The small-scale means used were shake flasks and various stirred tank reactors. Each production mean was continued for approximately 3 weeks.

The iron oxidation was initially determined to estimate the time required for 90% of the added Fe^{2+} to be oxidized. Thereafter, the oxidation was visually monitored. Once the culture turned strong orange (Figure 24), half of the medium in the cultivation vessel was replaced with fresh one. The iron oxidation was ≥ 90% after 2, 3 and 4 days in the shake flasks (at 35°C), glass stirred tank reactor (at 35°C) and bucket-type stirred tank (at 25°C) reactor, respectively. The highest redox of the bucket-type stirred tank reactors was ~570 mV (vs. Ag/AgCl). The pH in the reactors (~ pH 2) was higher than in the FBR (~pH 1.6) and in the inoculum production shake flasks (pH 1.3-1.7). The continuous feeding of the FBR enables the regulation of pH, which was not the case of the fed-batch reactors. The higher pH compared to the inoculum production shake flask was because of the AC addition to the laboratory-scale production means. Due to the elevated pH level and lower temperature in the bucket-type stirred tank reactors, the microbial community composition may have slightly changed during the biomass production. In total 14 L of AC bounded biomass was produced by the laboratory-scale methods during the 3-week cultivations.

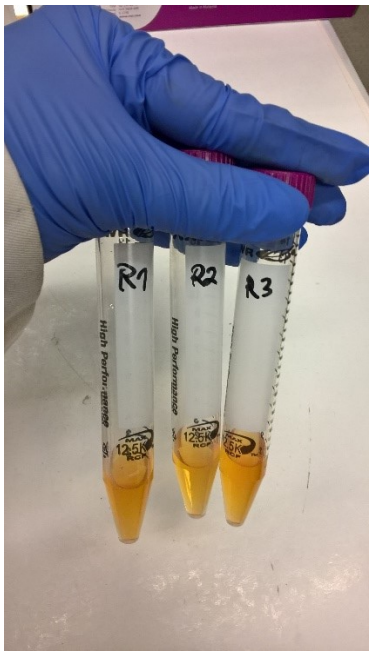


Figure 24: Liquid samples taken from the three bucket-type stirred tank reactors 4 days after media transfer.

6.2.2 Biomass production in semi-pilot scale

In the second-phase of biomass production, the 14 L AC bound biomass together with 80 L FBR effluent was used to start the semi-pilot scale operation with approximately 16% (v/v) inoculum, which is similar to that initially used in the laboratory-scale biomass production. The iron oxidation rate was 1.8 g/L/d during first 4 days of the semi-pilot reactor operation, but the oxidation rate decreased to 0.7-1.2 g/L/d after the first media transfer (day 6). The ferrous iron oxidation efficiency and the total soluble iron concentration (Figure 26) decreased in line being highest in the period until the first media transfer. The total soluble iron concentration decreased in the periods between media transfers until the 19th day when it became relatively stable. The DO was changing parallelly with the redox and remained most of the time between 4 and 8 mg/L. The peaks of Fe^{2+} (Figure 25) on days 6, 11, 13, 15, 19, and 22 shows the media transfer occasions. The decrease of Fe_{tot} at the end of operation was due to washing of the biomass with water (pH 2). The redox (Figure 26 a) increased with ferric production and was varied between 389 and 539 mV (vs. Ag/AgCl). The pH (Figure 26 b) was 2.0±0.3 during the operation, not taking into account the initial pH of 2.5.

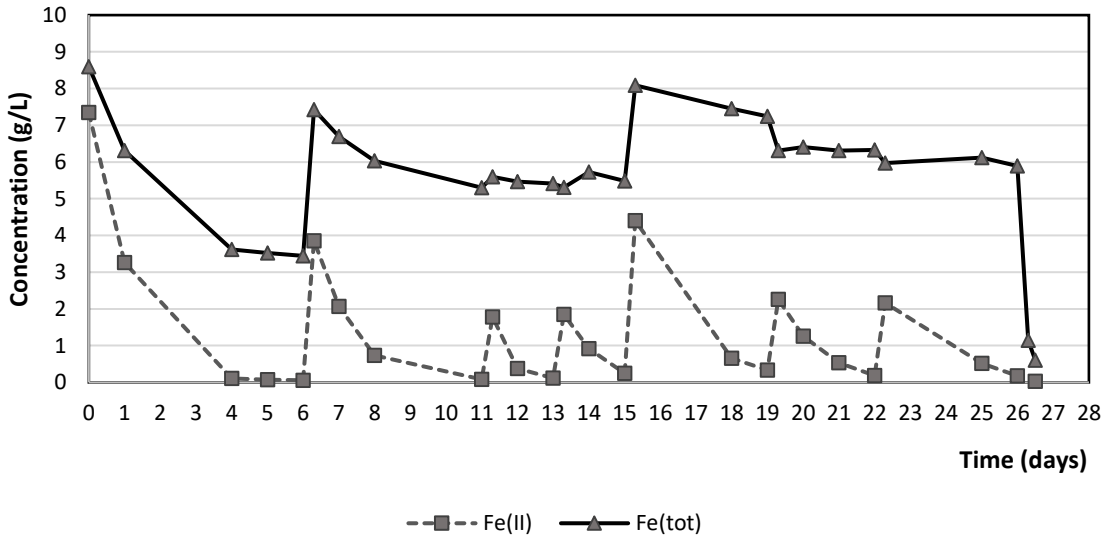
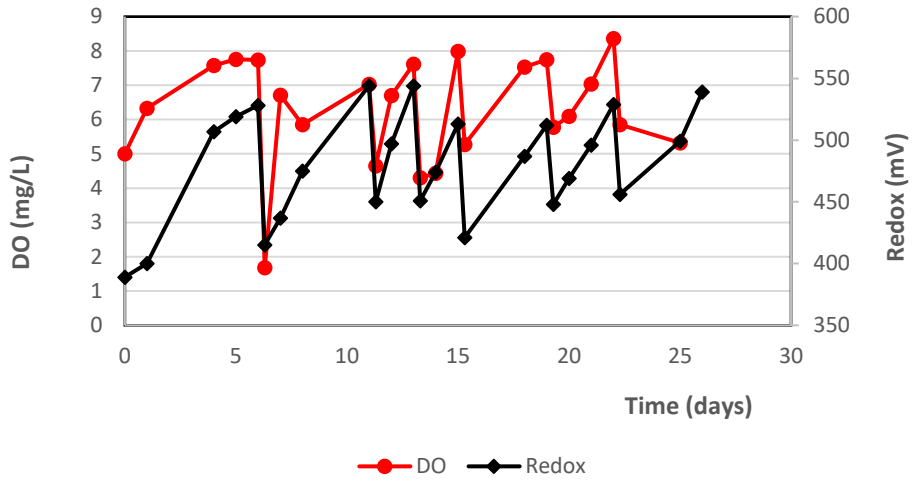
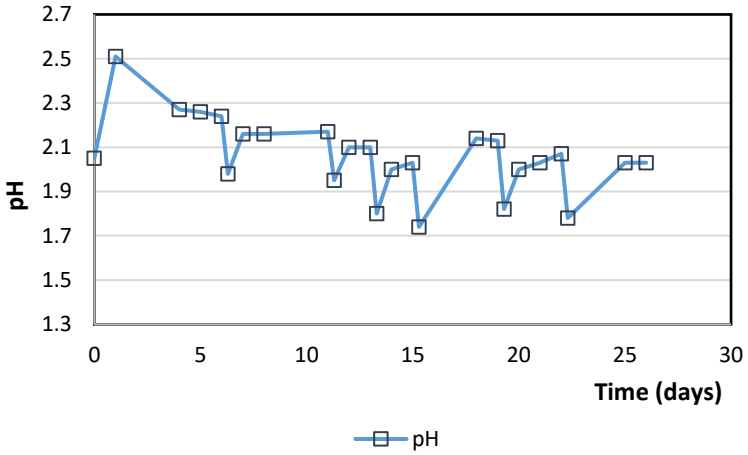


Figure 25: Changes in the soluble ferrous iron and total iron concentration throughout the 26 days biomass production in the semi-pilot reactor. The media transfer with 10 g/L Fe^{2+} took place on days 6 and 15, and with 5 g/L Fe^{2} on days 11, 13, 19 and 22.



a)



b)

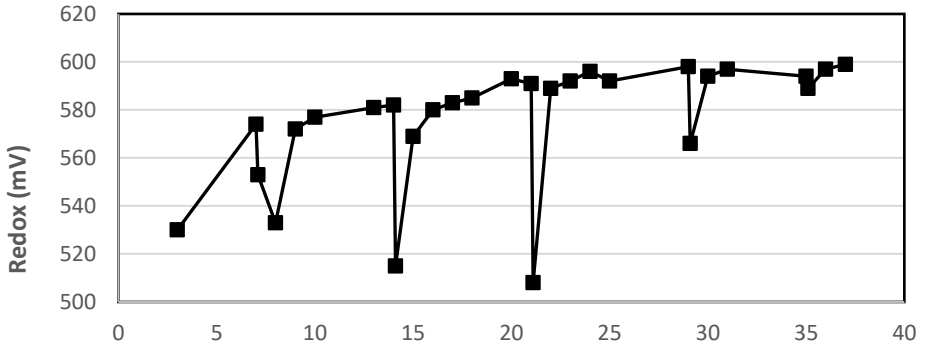
Figure 26: Changes of DO and redox potential (a) and pH (b) in the semi-pilot reactor throughout the 26-day operation.

Cultivation in the semi-pilot reactor for the 26-day cultivation period resulted in active AC bound biomass for starting the pilot-scale operation. The 252 L AC bounded biomass from the semi-pilot reactor was used as inoculum of the BIOMOre pilot reactor at Rudna mine, KGHM Polska Miedz, Poland.

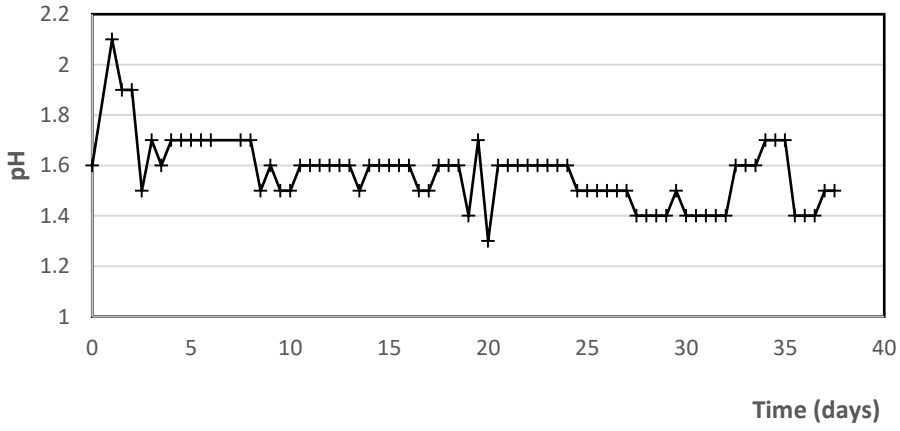
6.2.3 Maintenance of biomass in the pilot reactor

In this last phase of the biomass production, maintenance of the iron oxidizers until the start of deep *in-situ* bioleaching application was performed. The data for this study was collected from the BIOMOre chamber at the Rudna mine through 37 days period and obtained from Théodore Ineich, Hatch Ltd.

The variations of redox potential (Figure 27a) were insignificant due to the small amount of weekly ferrous iron additions. The small decreases in the redox were due to the weekly media transfers. Not considering the media transfer occasions, the redox potential remained nearby at same level (~590-600 mV vs. Ag/AgCl) after the day 19. The pH (Figure 27b) increased to 2.1 at the beginning of operation, but was adjusted back to 1.6 on day 5 and then maintained at that level. There was only small variations in the pH due to the automatic control. The temperature in the pilot reactor was at 28±2 °C and the aeration was ~100 L/min.



a)



b)

Figure 27: Changes of redox potential (a) and pH (b) in the pilot reactor. The pH was maintained at 1.6 and the variations in the figure are due to error of calibration or no contact with the liquid phase

The results showed that the pilot bioreactor filled with AC bound iron oxidizing biofilm is a good method to maintain the biomass activity. In this reactor the conditions can be maintained that allows the regulation of the iron oxidation rate that is important during *in situ* bioleaching operation. Due to the limited ferrous iron concentration, some biomass decay likely occurred during the pilot-reactor operation. Also, the microbial community composition may change with time due to the temperature levels being below 30°C, which is lower than in the laboratory-scale FBR (35 °C) from which the biomass originated.

A summary of iron oxidation rates obtained in the different cultivation systems used in this study is given in Table 6 and based on the data it can be seen that the highest rate (121 g/L/d) was achieved in the continuously-fed laboratory-scale FBR. The second highest rate was obtained in the shake flasks (1.7 g/L/d) and the third highest in the semi-pilot reactor (1.2 g/L/d). The lowest rates of iron oxidation were obtained in the pressure reactor. These results show the importance of using the adequate conditions (O_2 supply, temperature, Fe^{2+} addition, pH and pressure) during bioleaching operation.

Table 6: Summary of Fe^{2+} oxidation rates obtained at the different cultivation systems used in this study,

mean of bioleaching	**AC content (%)**	**working volume (L)**	**HRT (d)**	**Fe^{2+} oxidation (%)**	**Fe^{2+} oxidation rate (g/L/d)**
shake flasks	0	0.1	3	~95	1.7
*FBR**	~33	0.9	0.6	~99	121
pressure reactor (bar)					
+1	0	1.0	7	~73	0.59
+2	0	1.0	6	~97	0.73
+3	0	1.0	6	~99	0.78
semi-pilot reactor	~33	600	4	~92	1.2

*****The FBR results are not shown in this study and were obtained from Sarita Ahoranta, Tampere University of Technology

7. DISCUSSION

7.1 The effect of pressure on physicochemical leaching environment and iron oxidizing microorganisms

Application of elevated pressures has different effect on the abiotic and on the biotic iron oxidation. This study showed that with lower (+0.21, +0.41 and +1.47 bar) oxygen partial pressure the biotic iron oxidation dominates, while at higher levels (+3.15 and +6.3 bar) the abiotic does. There was linear increase of abiotic iron oxidation rate with increasing oxygen partial pressure. This result agrees well with the results of Chen and Thomson (2018) who demonstrated decrease of chemical iron oxidation efficiency when oxygen partial pressure was reduced.

The DO measurements of the abiotic experiments of this study showed similar results (9-14 mg/L) at oxygen partial pressure levels of +0.21, +0.63, +1.47 and +6.3 bar. At +2 bar pressure (p_{O2} = 0.41 bar) more variations (6-11 mg/L) and at +15 bar (p_{O2} = 3.15) higher peak (15 mg/L) than at the other pressure levels were observed. The DO measurements did not show actual DO concentration because part of the oxygen escaped from the samples during the sampling procedure. These DO losses were likely higher at higher pressures. The theoretical DO concentrations calculated by the equation (Section 4.1.3, Equation 11) from Tromans (1998) are 17, 35, 52, 121, 260 and 521 mg/L at +1, +2, +3, +7, +15 and +30 bar, respectively. The theoretical DO at 30 bar (521 mg/L) is already 65% of the oxygen that is needed (800 mg/L) to completely oxidize 5.6 g/L Fe(II) based on the stoichiometry of iron oxidation (Section 3.1, Equation 5) (Sand et al., 1995). The highest abiotic iron oxidation efficiency was 69% at +30 bar, which required 554 mg/L oxygen, based on the stoichiometry of iron oxidation. This means likely that more oxygen was dissolved from the gas phase as oxygen became utilized from the liquid phase by the oxidation reaction. Guezennec et al. (2016) tested DO concentrations between 4 and 17 mg/L and showed good oxidizing activity up to 13 mg/L and significant decrease in iron oxidation efficiency at 17 mg/L by a mixed culture (*L. ferriphilum, At. caldus and S. benefaciens*). They also reported that the DO concentration had lower effect on *L. ferriphilum* and *S. benefaciens* when the microorganisms were present as biofilm than on *At. caldus* that was rather growing in the liquid phase. The optimum DO concentration in a study by Wang et al. (2015) was 3.75 mg/L in their batch experiment with 10% refractory sulfide gold concentrate. The typically used minimum oxygen partial pressure during the chemical pressure oxidation with autoclave of refractory gold ore is 3.5 bar

(Fleming, 2009) which is much higher than the limit observed for biotic iron oxidation (0.63 bar) in this study.

The +40 bar experiment, in which the elevated pressure was induced using nitrogen gas leading to almost anaerobic conditions, demonstrated that the pressure itself and the rate of pressure increase/decrease (10 bar/min) is not inhibitory to the mixed acidophilic iron oxidizing culture (*L. ferriphilum and Sulfobacillus sp.)*. Davidson et al. (1981) tested the hydraulic compression of washed cell suspension (*T. ferrooxidans, T. thiooxidans and Thiobacillus* like bacterium) and reported no inhibition of iron oxidation up to 689 bar. Their rate of pressure decompression was 50 bar/s and they tested the iron oxidation right after the pressure experiment. Furthermore, Zhang et al. (2017) reported barotolerance and capability of ferric iron reduction by acidophilic microorganism (*Acidianus brierleyi, Thermoplasma acidophilum and Sulfolobus metallicus*) up to 100 bar under anaerobic conditions.

In the pressure experiments of this study, no inhibition of biological iron oxidation occurred at up to +3 bar (p_{O2}= 0.63 bar), while inhibition was observed at +7 bar (p_{O2}= 1.47 bar) up. Considering that the iron oxidizers tolerated the elevated pressure (+40 bar), it is likely that the inhibition was caused by the high oxygen partial pressure. The limiting p_{O2} on planktonic acidophilic microorganisms was considerably lower than that reported by Ahoranta et al. (2017b) for ore mineral surface attached acidophiles. They tested pressures up to 20 bar (p_{O2}= 4.2 bar) in the similar pressure reactor with sulphidic gold containing ore and mixed acidophilic culture (*At. ferrivorans, Ferrimicrobium acidiphilum and Sulfobacillus sp*.) and observed biotic iron oxidation up to 20 bar. This study showed that in aerobic conditions above atmospheric pressure the iron oxidation rate and efficiency of the iron oxidizing microorganisms decreased. The same trend was also reported by Ahoranta et al. (2017b).

In this work, the average iron oxidation rates at the pressure levels from +1 to +3 bar were 2.5 times lower than that was obtained from the shake flasks at atmospheric pressure. From the three non-inhibitory elevated pressures tested (+1, +2, +3 bar) the highest iron oxidation rate was observed at +3 bar (p_{O2}= 0.63 bar). Based on the results of the control shake flasks at atmospheric pressure, it was expected that closer to atmospheric pressure the biotic iron oxidation has higher rates. Despite the expectation, the +1 bar experiment had lower rate than the +2 and +3 bar experiments. The reason for the higher biotic oxidation rates can be that the initial oxygen addition with the +2 and +3 bar experiments were more adequate to meet the oxygen demand of the iron oxidation. Maybe with the +1 bar experiment the oxygen transfer rate from the gas-phase was not enough to fulfill the demand within the liquid-phase (du Plessis, 2007). Another explanation could be that more gas escaped during the sampling of +2 and +3 bar experiments than of the +1 bar

experiment. The gas escaped was each time replaced with fresh gas that had more oxygen than the one that has left from the pressure reactor. The minor gas loss of the +1 bar experiment, resulted in lower fresh gas addition and thus lower oxygen addition to the system.

Although it was not possible to monitor or control the DO concentration in the liquid media of the pressure reactor used in this study due to the high pressures, most of the bioleaching reactor designs enable the monitoring of DO concentration and the control of oxygen supply. The DO sensors are often connected online so the variations in the concentration can be remotely followed and the oxygen supply adjusted according to that (BIOMOre, 2016). The parameters can be fixed of the continuous modes of iron oxidation (like the FBR and future application of the pilot reactor) which enables steady-state conditions and good performance. In case of batch and fed-batch mode the process variables are not constant so to achieve efficient operation, the parameters cannot be fixed (Åkesson & Hagander, 1998; Court, 1988).

7.2 Effect of process scale-up on growth of iron oxidizing microorganisms

The results of this study showed that with increasing working volume the iron oxidation rate decreased in semi batch operation at atmospheric pressure. It was also recognized that the highest iron oxidation rate (121 g/L/h) was obtained by using continuously fed biofilm-based reactor (FBR). The pilot reactor will operate like an FBR so it is expected to perform similarly as the FBR of this study, i.e. at a high iron oxidation rate. Kinnunen & Puhakka (2004a) also used continuously fed FBR for their iron oxidation experiments with *L. ferriphilum* dominated culture and they obtained iron oxidation rate of 633 g/L/d (26.4 g/L/h). Although most of the cells attach to the surface of the activated carbon, the effluent from the FBR contained enough culture suspension for the start of laboratory-scale activated carbon-bound biomass production. Kinnunen & Puhakka (2004a) reported 10% of the cells in the liquid when using FBR with AC.

Each means used during the biofilm production had its own issues, which had effect on the iron oxidation efficiency. The shake flasks were too small, and the glass stirred tank reactor had evaporation. Once going to larger scale, the consequences of technical limitations became higher. Both the stirred tank reactors and semi-pilot reactor had too small agitators. Besides the agitator size, the semi-pilot reactor had a cubic shape, which resulted AC accumulation in the corners, from where it had to be manually removed.

Mixing is one of the key factors in bioleaching operations, because it is crucial for the maintenance of high level of oxygen transfer rate (Guezennec et al., 2017).

The pilot reactor is mixed by aeration, which seems to work well during culture maintenance phase. Later with higher ferrous iron load, higher volume of oxygen will be needed for the proper gas-liquid mass transfer, which can result in formation of jarosite and iron hydroxide precipitates potentially helping with the attachment of microorganisms (Kinnunen & Puhakka, 2004a). However, iron precipitates tend to block pumps and valves (for a review, see Nemati et al., 1998) so with the pilot reactor the same can happen with its air supplier.

After the last two media transfers in the pilot reactor, the redox potential had very minor variations and was around 600 mV (vs. Ag/AgCl) continuously as a consequence of ferrous iron limitation or simply the adjustment of the microbes to the new environment. For growth of the iron oxidizing microorganisms, large amount of Fe^{2+} need to be oxidized (Holmes & Bonnefoy, 2007). The Fe^{2+} is the primary energy source of various acidophile species (like the iron oxidizers of this study) and without its oxidation the reduction of $NAD^{+}(P)$ that is needed for CO_2 fixation cannot occur. CO_2 as sole carbon source is essential for the cell growth and anabolic processes thus its absence is inhibitory. (Dopson, 2012; D`Hugues, 2008).

Expect for the shake flasks and glass stirred tank reactor, the temperature was not maintained at 35°C in all of the biomass production systems and the pH in all means of biomass production was higher than in the FBR from which the inoculum originated from. Each iron oxidizing microorganism has its optimal pH and temperature. The initial iron oxidizing culture (*L. ferriphilum* dominated and containing some *Sulfobacillus sp.*) used in this study probably changed during the biomass scale up process. Maintenance of temperature at 35°C was possible with the shake flasks and glass stirred tank but this was not possible at the larger scale reactors (stirred tank reactor and semi-pilot reactor). *L. ferriphilum* has its optimum pH range between 1.3 and 1.8 and temperature between 30 and 37°C (Johnson, 2014; Schippers, 2007; Karavaiko et al., 2006). The pH values in the laboratory-scale stirred tank reactors were between 1.8 and 2.0 and the temperature around 25± 2°C. Especially in these higher pH levels, it is probable that some other acidophilic iron (or iron/sulfur) oxidizer specie appeared or even took over the culture. As an example, this could be *At. ferrooxidans* which has optimum pH between 1.8 and 2.5 and is common in stirred tank bioleaching (Johnson, 2014; Brandl, 2001; Krebs et al., 1997; Rawlings, 2002; Schippers, 2007). Considering the pH, another possible species would be *Sulfobacillus thermosulfidooxidans* which can grow at 20-60°C and prefers pH 2 (Johnson, 2014; Brandl, 2001; Rawlings, 2002; Schippers, 2007). Ahoranta et al. (2017b) had *Acidithiobacillus ferrivorans and Ferrimicrobium acidiphilum* besides

Sulfobacillus sp. in their stirred tank reactors operated at pH 2 and 27±1°C. Probably the most significant change of microbial culture in this study occurred during the semi-pilot reactor operation. In this reactor the pH was slightly above 2 and the temperature only 20± 2°C. In this semi-pilot reactor, the microbial culture remained likely similar as in the smaller scale stirred tank reactors.

More than 60% decrease in the total soluble iron concentration in the semi-pilot reactor was observed before any media transfer and it could be because some iron was accumulated onto the activated carbon. Activated carbon efficiently absorbs ferrous iron at pH 4.5-7.5 (102.96+/-4.49 and 100.94+/-19.02 mg, respectively) but does not adsorbs (-0.01+/-0.26 mg) ferrous iron in strongly acidic conditions (pH 1.5) (Chyka et al., 2001). Siabi (2003) reported fast (within 10 min) and efficient (98%) iron absorption onto the AC in circumneutral pH in batch reactor. The initial soluble iron concentration was 21 mg/L Fe^{2+} and 41 mg/L Fe_{tot}.

8. CONCLUSIONS

The following conclusions can be drawn based on this study:

- Growing microorganisms as biofilm rather than as cell suspension would potentially decrease the negative effects of elevated DO concentrations.

- Replacing high temperature with acidophilic microorganisms in pressure oxidation of refractory ore is a potential process application. This requires that relatively low oxygen partial pressures are maintained. The iron oxidizing microorganisms like *L. ferriphilum* attach to the surface of the ore, which probably increases DO tolerance. Testing the improvement of oxygen partial pressure tolerance of the iron oxidizing culture in biofilm is recommended.

- The maximum limit of oxygen partial pressure tolerance of the planktonic acidophilic iron oxidizers (*L. ferriphilum and Sulfobacillus sp.*) is between 0.63 and 1.47 bar. However, further studies are recommended so that the actual tolerance limit could be delineated.

- Deep *in situ* iron oxidation by the acidophilic culture used in this study is possible at least up to +3 bar, if enough oxygen to maintain the oxidation activity can be provided to the *in situ* environment.

- If oxygen is available, there is high abiotic iron oxidation rate in deep subsurface.

The conclusions and recommendations for the coming pilot study are as follows:

- Abiotic iron oxidation in deep *in situ* leaching requires high O_2 supply.

- Increasing the amount of ferrous iron addition to the pilot reactor is necessary for the improvement of the growth of the iron oxidizers.

- Adjustment of the pH to 1.3-1.8 and temperature to 30-37°C is recommended to maintain the dominance of *L. ferriphilum* in the pilot reactor.

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APPENDICES

Appendix 1: Chemicals

Chemical	Manufacturer
$Ca(NO_3)_2 \cdot 4\ H_2O$	Merk, Germany
$C_2H_4O_2$	Fisher Chemical, UK
CH_3CO_2H	Merk, Germany
$C_{12}H_8N_2 \cdot H_2O$	Merk, Germany
$C_{10}H_{16}N_2O_8$	ACRÓ Organics, Belgium
CH_3COONH_4	J.T. Baker, Holland
$C_{27}H_{34}I_2N_4$	Thermo Fisher Scientific,
$CoC_{l2} \cdot 6\ H_2O$	Merck, Germany
$CuSO_4 \cdot 5\ H_2O$	Merk, Germany
Fe	Fluka Analytical, Switzerland
$FeC_{l3} \cdot 6\ H_2O$	Sigma Aldrich, USA
$FeSO_4 \cdot 7\ H_2O$	VWR Chemical, Germany
H_3BO_3	Merk, Germany
HCl (37%)	VWR Chemical, France
HNO_3 (69 %)	BDH, England
H_2SO_4 (95-97 %)	Fisher Chemical, USA
KCl	Sigma Aldrich, USA
K_2CO_3	J.T. Baker, Holland
K_2HPO_4	VWR, Belgium
$MgSO_4 \cdot 7\ H_2O$	Merk, Germany
$MgSO_4$	ACRÓ Organics, Germany
$MnSO_4 \cdot 4\ H_2O$	Merk, Germany
NaCl	Merk, Germany
$Na_2MoO_4 \cdot 2\ H_2O$	J.T.Baker, Holland
$Na_2P_2O_7$	Merck, Germany
Na_2SeO_4	Sigma-Aldrich, Germany
$Na_2SO_4 \cdot 10\ H_2O$	Merck, Germany
$(NH_4)_2HPO_4$	ACRÓ Organics, Spain
$(NH_4)_2SO_4$	Sigma Life Science, USA
Syto®9	Thermo Fisher Scientific, USA
$ZnSO_4 \cdot 7\ H_2O$	VWR, Belgium

Appendix 2: AGA Technical air used with the pressure experiments (AGA, 2018)

A Member of The Linde Group | AGA	KÄYTTÖTURVALLISUUSTIEDOTE	Sivu : 1
		Julkaisu no : 7 - 1
		Päiväys : 20 / 1 / 2015
		Korvaa : 6 / 7 / 2012
TEKNINEN ILMA		IG 000AC

2.2 : Palamattomat, myrkyttömät kaasut

Varoitus

KOHTA 1. Aineen tai seoksen ja yhtiön tai yrityksen tunnistetiedot

1.1. Tuotetunniste

Kauppanimi : TEKNINEN ILMA

Käyttöturvallisuustiedote nro : IG 000AC

1.2. Aineen tai seoksen merkitykselliset tunnistetut käytöt ja käytöt, joita ei suositella

Merkitykselliset tunnistetut käytöt : Teollinen ja ammattimainen. Tee riskianalyysi ennen käyttöä. Testikaasu / Kalibrointikaasu. Laboratoriokäyttö. Ota yhteyttä toimittajaan, jos tarvitset käyttötietoja.

Käytöt, joita ei suositella : Kuluttajien käyttöön.

1.3. Käyttöturvallisuustiedotteen toimittajan tiedot

Yrityksen tunnistetiedot : Oy AGA Ab
Itsehallintokuja 6
02600 ESPOO, Finland

Puh. 010 2421
info@fi.aga.com
www.aga.fi

1.4. Hätäpuhelinnumero

Hätäpuhelinnumero : Myrkytystietokeskus (24h): 09 471 977 (suora) tai 09 4711 (vaihde)

KOHTA 2. Vaaran yksilöinti

2.1. Aineen tai seoksen luokitus

Vaaraluokka ja kategoriakoodi asetuksen 1272/2008/EY (CLP) mukaan

- Fysikaaliset vaarat : Paineen alaiset kaasut - puristetut kaasut - Varoitus - (CLP : Press. Gas Comp.) - H280

Luokitus 67/548/EY tai 1999/45/EY

: Ei luokiteltu vaaralliseksi aineeksi / seokseksi.

2.2. Merkinnät

Merkinnät asetuksen 1272/2008/EY (CLP) mukaan

- Varoitusmerkit

- Varoitusmerkin koodi : GHS04

- Huomiosana : Varoitus

KOHTA 2. Vaaran yksilöinti /...

- **Vaaralausekkeet** : H280 - Sisältää paineen alaista kaasua; voi räjähtää kuumennettaessa.
- **Turvalausekkeet**
  - **Varastointi** : P403 - Varastoi paikassa, jossa on hyvä ilmanvaihto.

2.3. Muut vaarat

: Ei mitään.

KOHTA 3. Koostumus ja tiedot aineosista

3.1. Aine / 3.2 Seos

Seos.

Aineosan nimi		Pitoisuus	CAS-nro EY-nro Indeksinumero Rekisteröintinumero	Luokitus(DSD)	Luokitus(CLP)
Happi	:	20,9 %	7782-44-7 231-956-9 008-001-00-8 * 1	O; R8	Ox. Gas 1 (H270) Press. Gas Comp. (H280)
Typpi	:	79,1 %	7727-37-9 231-783-9 ---- *1	Ei luokiteltu (DSD)	Press. Gas Comp. (H280)

Ei sisällä muita aineosia tai epäpuhtauksia, jotka vaikuttavat tuotteen luokitukseen.
* 1: Mukana Annex IV/V REACH, ei rekisteröimisen alainen.
* 2: Rekisteröinnin määräaika ei umpeutunut.
* 3: Rekisteröintiä ei vaadita: Ainetta valmistettu tai maahantuotu < 1t/v.
R-lausekkeet kokonaisuudessaan katso luku 16. H-lausekkeet (vaaralausekkeet) kokonaisuudessaan katso luku 16.

KOHTA 4. Ensiaputoimenpiteet

4.1. Ensiaputoimenpiteiden kuvaus

- **Hengitys** : Käytä paineilmalaitetta ja siirrä uhri raittiiseen ilmaan. Pidä uhri lämpimänä ja levossa. Kutsu lääkäri paikalle. Anna tekohengitystä, mikäli hengitys on pysähtynyt.
- **Ihokosketus** : Tällä tuotteella ei tiedetä olevan haittavaikutuksia.
- **Silmäkosketus** : Tällä tuotteella ei tiedetä olevan haittavaikutuksia.
- **Nieleminen** : Nielemistä ei pidetä todennäköisenä altistumistienä.

4.2. Tärkeimmät oireet ja vaikutukset, sekä välittömät että viivästyneet

: Oireita voivat olla huimaus, päänsärky, pahoinvointi ja koordinaatiokyvyn menetys.
Katso kohta 11.

4.3. Mahdollisesti tarvittavaa välitöntä lääketieteellistä apua ja erityishoitoa koskevat ohjeet

: Hakeudu lääkärin hoitoon.